A system for improved production titers in fermentations

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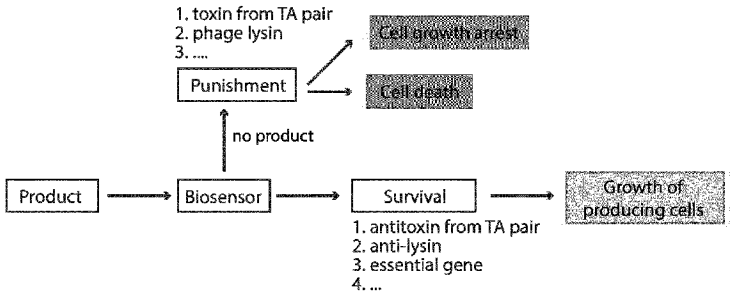
Figure 1



(57) Abstract: The invention provides a genetically modified micro-organism for intracellular biosynthesis of a cellular metabolite, comprising a synthetic error correction system having a penalty gene, whose expression leads to arrested growth or cell death (e.g. a toxin gene) in combination with a survival gene, whose expression provides an antidote that restores cell viability and normal growth (e.g. a cognate antitoxin gene). Alternatively, the system has a survival gene, alone, whose expression is essential for growth (i.e. essential gene). The synthetic error correction system further comprises a biosensor, whose function is to induce expression of the survival gene which leads to cell growth, only, when the cell produces a pre-defined level of a given metabolite. The invention further encompasses: a method for producing the genetically modified micro-organism; a method for producing a cellular metabolite with the genetically modified micro-organism; and use of the genetically modified micro-organism for producing a cellular metabolite.

TITLE: A system for improved production titers in fermentations

Technical field of the invention

The invention provides a genetically modified micro-organism for intracellular biosynthesis of a cellular metabolite, comprising a synthetic error correction system having a penalty gene, whose expression can either lead to arrested growth or cell death (e.g. a toxin gene) in combination with a survival gene, whose expression provides an antidote that can restore cell viability and normal growth (e.g. a cognate antitoxin gene). Alternatively, the system has a survival gene, alone, whose expression is essential for growth (i.e. essential gene). Additionally, the synthetic error correction system has a biosensor, whose function is to induce expression of the survival gene leading to cell growth, only, when the cell produces a pre-defined level of a given metabolite. The invention further encompasses: a method for producing the genetically modified micro-organism; a method for producing a cellular metabolite with the genetically modified micro-organism; and use of the genetically modified micro-organism for producing a cellular metabolite.

Background of the invention

An increasing share of the world's chemical production relies on microorganisms or mammalian cells that are genetically engineered to function as cell factories, and tailor-made for the biosynthesis of a given molecule. Production processes, employing these cell-factories, are typically initiated from a starter culture of a small number of cells of a production organism, which go through a phase of growth and expansion of cell numbers in large fermentation tanks (up to 30,000 L volume). In some setups, production of a given molecule proceeds both during the growth phase and during a subsequent period (batch and fed-batch cultures). Alternatively, in order not to waste resources associated with cleaning and growing up a new batch, production is continuous. A chemostat fermentor allows a production organism to be grown in a fermentation broth that is constantly diluted, thus tapping product and cells from the culture, while replenishing with fresh nutrient medium. On an industrial scale, such production processes may continue operation for 1-2 months before starting a new culture in a clean

tank. The fermentation processes and equipment used in this industry are very similar, both for the production of a wide range of commodity small molecules and for therapeutic proteins, and consequently these processes are subject to similar problems.

In particular, the appearance of non-producing cells (unable to produce the product molecule) is commonly observed, especially when the production run is for extended periods of time (chemostat). Such non-producing cells within an industrial fermentation are highly undesirable, as they consume nutrients, oxygen and space. Furthermore, non-producing cells can have a selective advantage over producing cells, and may as such grow faster. In a growing cell culture, such improvements in fitness can lead to significant out-competition of the producing cells over time. This drift from the optimal production state is an eventual reason for discarding the fermentation broth and spending resources on cleaning, sterilization, not to mention nutrients, to replenish the fermentation tank with new, producing organisms. Such non-producing cells originate from genetic mutations that arise in the cells of an original producing organism undergoing many growth divisions.

Since the occurrence of genetic mutations in cells of a production organism that lead to a loss of product formation by the cells during a production run, cannot be avoided, there is a need for methods for eliminating or slowing the growth of non-producing cells in the production. Preferably, such methods of elimination are sufficiently effective that they prevent the observed drift from the production state, and thereby prolong the life-time of an industrial fermentation.

If the system employs a penalty gene alone (and no antidote), then an "OFF" type sensor is required. If the system employs a survival gene alone (e.g. essential genes) a metabolite-linked "ON" type sensor is needed as in the first scenario.

Summary of the invention

The invention provides a genetically modified microbial cell for intracellular biosynthesis of a cellular metabolite comprising:

a) a first nucleic acid molecule wherein the transcription and/or translation of said molecule yields a biosensor capable of binding said cellular metabolite to form a complex; and

any one selected from the group consisting of ((b), (c) and (d)):

b) a second nucleic acid molecule comprising a coding sequence encoding a first protein required for cell growth and/or survival, wherein the second nucleic acid molecule is operably linked to a first promoter; wherein expression of said first protein encoded by said second nucleic acid molecule is induced when said biosensor and said cellular metabolite form a complex;

c) a second nucleic acid molecule comprising a coding sequence encoding a first protein required for cell growth and/or survival, wherein the second nucleic acid molecule is operably linked to a first promoter; and

a third nucleic acid molecule encoding a second protein that is toxic for cell growth and/or survival, wherein said third nucleic acid molecule comprises a coding sequence operably linked to a second constitutive promoter; wherein expression of said first protein encoded by said second nucleic acid molecule is induced when said biosensor and said cellular metabolite form a complex; and

d) a second nucleic acid molecule encoding a protein that is toxic for cell growth and/or survival, wherein said second nucleic acid molecule comprises a coding sequence operably linked to a promoter; wherein expression of said protein is prevented when said biosensor and said cellular metabolite form a complex;

whereby arrest of growth and /or death of said cell due to an absence of complex formation does not depend on externally supplied growth inhibitor or growth retardant.

According to one embodiment of the genetically modified microbial cell of invention comprising the features of (a) and (b), the first promoter is an inducible promoter, and the first protein (encoded by the second nucleic acid molecule) is essential for growth of the cell.

According to a further embodiment, the genetically modified microbial cell of the invention comprises the features of (a) and (c), wherein:
said first promoter linked to said second nucleic acid molecule is inducible, and said second protein (encoded by the third nucleic acid molecule) is a toxin; and said first protein (encoded by the second nucleic acid molecule) is an anti-toxin protein cognate to said toxin protein;
and said biosensor is a transcription factor capable of binding to said metabolite to form a complex, and wherein said complex is capable of binding to said inducible promoter so as to induce expression of the first protein.

According to a further embodiment, the genetically modified microbial cell of the invention comprises the features of (a) and (c), wherein:
said second protein (encoded by the third nucleic acid molecule) is a toxin; and wherein:
said first protein (encoded by the second nucleic acid molecule) is an anti-toxin cognate to said toxin;
said first nucleic acid molecule is operably linked to said second nucleic acid molecule upstream to its coding sequence and is operably linked downstream of the first promoter, and wherein the first promoter is a constitutive promoter, and
said biosensor obtained on transcription of said first nucleic acid molecule is a riboswitch capable of binding to said metabolite to form a complex.

According to a further embodiment, the genetically modified microbial cell of the invention comprises the features of (a) and (d), wherein:
said protein (encoded by the second nucleic acid molecule) is a toxin; and said promoter is inducible; and
and said biosensor is a transcription factor capable of binding to said metabolite to form a complex, and wherein said complex is capable of binding to said inducible promoter so as to induce expression of the protein.

According to a further embodiment, the genetically modified microbial cell of the invention comprises the features of (a) and (d), wherein:
said protein (encoded by the nucleic acid molecule) is a toxin;

said first nucleic acid molecule is operably linked to said second nucleic acid molecule upstream of its coding sequence and is operably linked downstream of the first promoter, and wherein the first promoter is a constitutive promoter, and wherein
said biosensor obtained on transcription of said first nucleic acid molecule is a riboswitch capable of binding to said metabolite to form a complex.

According to a further embodiment of the genetically modified microbial cell of the invention, the cellular metabolite is selected from the groups consisting of: isoprenoid(s), vitamin(s), carboxylic acid(s), amino acid(s), fatty acid(s), alcohol(s), and polyketide(s).

The invention further provides a method of genetically modifying a microbial cell for the biosynthesis of a metabolite comprising the steps of introducing into the cell:
a nucleic acid molecule encoding a toxin operably linked to a constitutive promoter;
a nucleic acid molecule encoding an anti-toxin cognate to the toxin, wherein the molecule is linked to an inducible promoter; and
a nucleic acid molecule wherein the transcription and/or translation of said molecule yields a biosensor capable of binding to the metabolite;
wherein expression of said antitoxin is induced when said biosensor and said cellular metabolite form a complex and bind to said inducible promoter; and
wherein arrest of growth and /or death of said cell due to an absence of complex formation does not depend on externally supplied growth inhibitor or growth retardant.

The invention further provides a method for producing a biosynthetic metabolite comprising the steps of:
providing a genetically modified microbial cell according to any one embodiment of the invention,
introducing the genetically modified microbial cell into a cultivation medium comprising a substrate for production of said metabolite, and
optionally cultured in a culture vessel by continuous culture for a period of at least 24 cell multiplications; and then

recovering metabolite produced by said culture,
wherein a lack of metabolite production in said genetically modified microbial cell or progeny cell thereof attenuates multiplication of said cell as compared to a non-genetically modified parent cell from which said modified microbial cell was derived.

The invention further includes the use of a genetically modified microbial cell according to any one embodiment of the invention, for producing a biosynthetic metabolite, wherein a lack of metabolite production in said genetically modified microbial cell or progeny cell thereof attenuates multiplication of said cell as compared to the metabolite producing genetically modified microbial cell.

Description of the invention

FIGURES

Figure 1. Cartoon of the general concept of synthetic error-correction.

Figure 2. Cartoon of L-arabinose-addiction regulated TA-system Dual expression TA gene construct comprising the toxin gene *yoeB* whose expression is regulated by the p_{Lac} promoter; and the antitoxin gene *yefM* whose expression is regulated by the p_{BAD} promoter, and the *araC* L-arabinose sensor gene.

Figure 3. Growth dependency through the *yoeB-yefM* system on induction of respectively toxin and antitoxin, as measured by cell density (OD_{600nm}) as a function of time (hr).
A) Cell growth in the absence of an inducer (L-arabinose) of antitoxin expression, and with a supply of IPTG (I), a toxin expression inducer, at a range of concentrations rising from 0, 0.005, 0.025, 0.1, 0.5, 1.5 mM IPTG, (corresponding to 0I to 5I, respectively).
B) Cell growth in the presence of an inducer (0.1 %L-arabinose) of antitoxin expression, and with a supply of IPTG (I), a toxin expression inducer, at a range of concentrations rising from 0, 0.005, 0.025, 0.1, 0.5, 1.5 mM IPTG, (corresponding to 0I to 5I, respectively). Growth curves are averages (n = 3).

Figure 4. Cartoon of mevalonate addiction regulated TA-system Dual expression TA gene construct comprising the toxin gene *yoeB* whose expression is regulated by the p_{Lac} promoter; and the antitoxin gene *yefM*, whose expression is regulated by the p_{BAD} promoter, and the mutated *araCmev* mevalonate sensor gene with 4 point mutations.

Figure 5. Cartoon of the operon encoding two mevalonate biosynthesis pathways from acetyl-CoA:
A) pMevTC operon comprising *E. coli atoB* encoding an acetyl-CoA acetyltransferase; *Saccharomyces cerevisiae ERG13* encoding an HMG-CoA synthase, and *tHMGR*, a truncated version of *Saccharomyces cerevisiae HMGR*, encoding an HMG-CoA reductase;
B) pMEV7C operon comprising *E. coli atoB*; a *Lactococcus lactis* gene *mvaS* encoding an HMG-CoA synthase, and a *Lactococcus lactis mvaE* gene encoding a HMG-CoA reductase; and
C) mevalonate biosynthetic pathway: the *atoB* encoded acetoacetyl- CoA thiolase catalyzes the formation of acetoacetyl-CoA from two molecules of acetyl-CoA; the *ERG13/mvaS* encoded HMG-CoA synthase, which creates 3-hydroxy-methylglutayl-CoA (HMG-CoA) by a condensation reaction between acetoacetyl-CoA and another molecule of acetyl-CoA; and *tHMGR/mvaE* encoded HMG-CoA reductase which converts HMG-CoA to mevalonate.

Figure 6. Cancelling the fitness advantage of non-producing cells. **A)** Cell growth over time (measured by cell density at OD_{600nm}) of *E. coli* at 30 ^{o}C in 2xYT medium, comprising the plasmid (pMEV7C) for 'high' level mevalonate synthesis and the control plasmid (pMevT5c) for no mevalonate synthesis.
B) Growth curves of strains harbouring the same pathway plasmids as well as the mevalonate-TA correction system.

Figure 7. Enhancing mevalonate production in fermentation populations using the TA system to eliminate non-producing cells. Histogram showing mevalonate (MVA) levels produced by mixture cultures with the indicated percentage (x-axis) of producing cells among non-producing cells. Producing cells comprised the mevalonate production plasmid (pMEV7C) while non-producing cells comprised the control plasmid (pMevT5c) with the inactivated

production pathway. The cells were tested with/without the plasmid expressing the mevalonate-TA system for "correction" (pBAM-TA5).

Figure 8. Cartoon showing a cloning and strain construction strategy for addiction through essential genes. The metabolite-responsive promoter is introduced in the genome of the host strain, replacing the native promoter of an essential gene (optionally operon). Upstream and downstream homologous regions (HR) guide replacement of native DNA with the responsive promoter and a kanamycin resistance gene for selection of genomic DNA insertion. Using a variable ribosome binding site (RBS) with redundant nucleotide, a wider range of responses can be screened in order to identify a host strain, which has become addiction an internally produced metabolite.

Figure 9. Cell growth controlled by the L-arabinose-addiction regulated essential gene system. Cell growth over time (measured by cell density at OD_{600nm}) of *E. coli* comprising the essential gene operon *folP/glmM* driven by the p_{BAD} promoter were cultivated at 37 °C in 2xYT growth medium supplemented with 0% or 0.25% L-arabinose. Growth curves are averages (n = 3). Error bars denote standard error.

Figure 10. Growth of *E. coli* strains addicted to internal production of mevalonate. Cell growth over time (measured by cell density at OD_{600nm}) of *E. coli* strains engineered to be mevalonate-addicted through the essential gene operon *folP/glmM*, comprising inoculums of the 100 % mevalonate producing strain (*e3.9*) and inoculum mixtures with the given percentages of the mevalonate producing strain (*e3.9*) and a mevalonate pathway non-producing strain (*e3.8*). Growth curves are averages (n = 3). Error bars denote standard error.

Figure 11. Fitness cost to non-addicted control cells of supplemented triacetic acid lactone (TAL). Growth of *E. coli* control strain e3.16CON, measured as increase in cell density (OD_{630nm}) over time, in 2xYT medium (with kanamycin and spectinomycin) supplemented with respectively 0, 2 and 20 mM triacetic acid lactone at 37 deg. C in 200 µL microtiter plates with continuous shaking. All wells were inoculated with the same number of cells. Error bars depict the standard error of the mean (n = 3).

Figure 12: Triacetic acid lactone-addicted growth of *E. coli* strain e3.16#5 in 2xYT medium (with kanamycin and spectinomycin) supplemented with respectively 0, 2 and 20 mM triacetic acid lactone (TAL), measured as increase in cell density (OD_{630nm}) over time. Cells of the strain were grown at 37 deg. C in 200 µL microtiter plates with continuous shaking. All wells were inoculated with the same number of cells. Error bars depict the standard error of the mean (n = 3).

Figure 13: Fitness cost to non-addicted control cells of supplemented salicylic acid. Growth of *E. coli* e3.18CON, measured as increase in cell density (OD_{630nm}) over time, in 2xYT medium (with kanamycin and spectinomycin) supplemented with respectively 0 or 5 mM salicylic acid and grown at 37 deg. C in 200 µL microtiter plates with continuous shaking. All wells were inoculated with the same number of cells. Error bars depict the standard error of the mean (n = 3).

Figure 14: The salicylic acid-addicted growth of *E. coli* e3.18#1, measured as increase in cell density (OD_{630nm}) over time, cultured in 2xYT medium (with kanamycin and spectinomycin) supplemented with respectively 0 or 5 mM salicylic acid and grown at 37 deg. C in 200 µL microtiter plates with continuous shaking. All wells were inoculated with the same number of cells. Error bars depict the standard error of the mean (n = 3).

Figure 15: Fitness cost to control cells of supplemented benzoic acid. Growth of *E. coli* e3.22CON, measured as increase in cell density (OD_{630nm}) over time, in 2xYT medium (with kanamycin and spectinomycin) supplemented with respectively 0 or 5 mM benzoic acid and grown at 37 deg. C in 200 µL microtiter plates with continuous shaking. All wells were inoculated with the same number of cells. Error bars depict the standard error of the mean (n = 3).

Figure 16: Benzoic acid-addicted growth of *E. coli* e3.22#4, measured as increase in cell density (OD_{630nm}) over time, in 2xYT medium (with kanamycin and spectinomycin) supplemented with respectively 0 or 5 mM benzoic acid and grown at 37 deg. C in 200 µL microtiter plates with continuous shaking.

All wells were inoculated with the same number of cells. Error bars depict the standard error of the mean (n = 3).

Figure 17. Cartoon of a DNA construct comprising a TPP-addiction riboswitch-regulated TA system.

Figure 18. Cell growth controlled by the thiamine pyrophosphate (TPP)-responsive riboswitch linked to a TA system. Cell density following 15 hours of incubation at 37 °C. (measured by cell density at OD_{600nm}) of *E. coli* comprising TPP-responsive riboswitch controlling the translational rate of the antitoxin, YefM, and a constitutively expressed toxin, YoeB (0μM IPTG) or induced toxin by LacI with IPTG inducer (5, 50 and 500 μM IPTG). Cell density measured in presence and absence of 500 μM TPP added to the growth medium.

Figure 19. Improvement with metabolite-addiction system following long-term culture. Concentration of mevalonate (MVA) accumulated in *E. coli* XL1 featuring the same MVA pathway plasmid following a total of 55 cell generations. Strain *e3.9* unlike *pe1* further features a chromosomal change in the promoter of an essential gene operon rendering its expression dependent on the product, MVA. Error bars denote standard deviation of biological replicates (n=3).

Abbreviations and terms:

gi number: (genInfo identifier) is a unique integer which identifies a particular sequence, independent of the database source, which is assigned by NCBI to all sequences processed into Entrez, including nucleotide sequences from DDBJ/EMBL/GenBank, protein sequences from SWISS-PROT, PIR and many others.

Amino acid sequence identity: The term "sequence identity" as used herein, indicates a quantitative measure of the degree of homology between two amino acid sequences of substantially equal length. The two sequences to be compared must be aligned to give a best possible fit, by means of the insertion of gaps or alternatively, truncation at the ends of the protein sequences. The sequence identity can be calculated as ((Nref-Ndif)100)/(Nref), wherein Ndif is the total number of non-identical residues in the two sequences when aligned and wherein Nref is the number of residues

in one of the sequences. Sequence identity can alternatively be calculated by the BLAST program e.g. the BLASTP program (Pearson W.R and D.J. Lipman (1988)) (www.ncbi.nlm.nih.gov/cgi-bin/BLAST). In one embodiment of the invention, alignment is performed with the sequence alignment method ClustalW with default parameters as described by Thompson J., et al 1994, available at http://www2.ebi.ac.uk/clustalw/.

Preferably, the numbers of substitutions, insertions, additions or deletions of one or more amino acid residues in the polypeptide as compared to its comparator polypeptide is limited, i.e. no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 substitutions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 insertions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 additions, and no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 deletions. Preferably the substitutions are conservative amino acid substitutions: limited to exchanges within members of group 1: Glycine, Alanine, Valine, Leucine, Isoleucine; group 2: Serine, Cysteine, Selenocysteine, Threonine, Methionine; group 3: Proline; group 4: Phenylalanine, Tyrosine, Tryptophan; Group 5: Aspartate, Glutamate, Asparagine, Glutamine.

Native gene: endogenous gene in a microbial cell genome, homologous to host micro-organism.

Biosensor: Suitable biosensor effectors (small molecules or metabolites detectable by a sensor) and their sensors can be found in the RegPrecise Collection of Manually Curated Inferences of Regulons in Prokaryotic Genomes, available at:
http://regprecise.lbl.gov/RegPrecise/collections_effector.jsp

Cell multiplication: process whereby cells multiply in number, as one cell divides into two cells, these two cells both divides to become four cells, four cells become eight, eight cells become sixteen etc. Each round of cell multiplication, by cell division, yields a new cell generation.

Cognate: in the context of Toxin-Antitoxin systems; an antitoxin protein interacts with its cognate toxin to neutralise the activity of the toxin.

Fitness cost: reduction in growth rate of a microbial cell e.g. due to producing a small molecule or metabolite (including a protein), measured relative to the growth rate of a reference microbial cell where production is inactivated. Fitness cost of a metabolic pathway can also be observed as an

increase in growth lag phase, again relative to a reference cell where this pathway is inactivated.

Growth inhibitor/retardant of external origin: in the context of the invention, a growth inhibitor/retardant is a component of external origin that can inhibit or retard the growth of a micro-organism (such as a toxin or antibiotic), that is not produced by the micro-organism itself, but instead is supplied to the micro-organism either by addition to or its presence in the growth medium or environment in which the micro-organism is cultured.

Operably linked: a gene (nucleic acid molecule comprising a coding sequence) is operably linked to a promoter when its transcription is under the control of the promoter and where transcription results in a transcript whose subsequent translation yields the product encoded by the gene. Similarly a first nucleic acid molecule encoding a riboswitch may be operably linked to a second nucleic acid molecule upstream of its coding sequence; and also operably linked downstream of a promoter; whereby transcription of the downstream first nucleic acid molecule comprising the riboswitch linked to the second nucleic is under the control of the promoter and results in a transcript; and whereby translation of the transcript so produced (comprising the riboswitch and coding sequence) is under the control of the riboswitch.

Toxic: in the context of the invention a protein is defined as toxic if arrests or limits cell growth and/or prevents cell survival; e.g. a protein toxin.

Detailed description of the invention

The present invention aims to prolong the productive life-time of an industrial fermentation by preventing the observed drift of a population of cells in an industrial fermentation from a productive state to a non-productive state. This drift typically arises as a result of spontaneous genetic mutations in cells of the production organism during continuous growth, and where a competitive advantage of a non-producing mutant favors its proliferation.

I: A genetically modified microbial cell comprising a synthetic error correction system controlled by a metabolite biosensor

The invention is based on the general concept of a synthetic error correction system which is illustrated in figure 1. This system uses either a penalty gene, whose expression can either lead to arrested growth or cell death (e.g. a toxin gene) in combination with a survival gene, whose expression provides an antidote that can restore cell viability and normal growth (e.g. a cognate antitoxin gene). Alternatively, the system uses a survival gene, alone, whose expression is essential for growth (i.e. essential gene).

The second key component of the system is a biosensor, whose function is to induce an appropriate gene regulation response leading to cell growth only when the cell produces a pre-defined level of a given metabolite. Cell survival and growth is said to be "addicted" to the presence of its metabolite i.e. to its addiction molecule. The functional properties of the biosensor depend on the type of gene regulation response required. If the system employs a penalty gene (such as a toxin gene) in combination with a survival gene (a cognate anti-toxin gene), then cell survival requires an "ON" type sensor linked to the expression of the survival gene. If the system employs a penalty gene alone (and no antidote), then an "OFF" type sensor is required. If the system employs a survival gene alone (e.g. essential genes) a metabolite-linked "ON" type sensor is needed as in the first scenario.

Accordingly, the fate of a cell, namely its survival and growth versus its arrested growth and eventual death, when employing this system, is determined by its continued production of a given metabolite, which is the product, or a close biosynthesis intermediate of the product of the industrial fermentation. A key feature of the synthetic error correction system in cells of the invention, is that the penalty executed by means of the error correction system in non-producing cells is realized by the expression of the penalty gene(s) and/or the failure to express the survival gene(s) (i.e. the expression of these genes and the cellular products thereof is both necessary and sufficient for achieving error correction). Accordingly, the arrest of growth or death of non-producing cells of the invention does not require the presence or addition of externally supplied compounds (e.g. toxins or antibiotics) for the execution of the penalty. This is an advantageous feature of the present invention, since the use of antibiotics or other growth retardants in the cultivation medium during industrial scale microbial fermentation would

compromise the economics, biosafety and stability of production. The invention provides a genetically modified microbial cell for use in the intracellular biosynthesis of a cellular metabolite, comprising the above described synthetic error correction system. The genetically modified microbial cell comprises at least: a first nucleic acid molecule wherein the transcription and/or translation of said molecule yields a biosensor capable of binding the cellular metabolite to form a complex; and a second nucleic acid molecule comprising a coding sequence operably linked to a promoter, and encoding a protein required for cell growth and/or survival; and optionally a third nucleic acid molecule comprising a coding sequence operably linked to a promoter, and encoding a protein that inhibits the growth and/or survival of the cell. The expression of the protein encoded by the second nucleic acid molecule is induced when the biosensor and the cellular metabolite form a complex. Various embodiments of the genetically modified microbial cell of the invention are described below:

II: A genetically modified microbial cell comprising a toxin-antitoxin system controlled by a metabolite biosensor

According to a first embodiment, the genetically modified microbial cell, which is for use in the intracellular biosynthesis of a given cellular metabolite, is a cell comprising a toxin-antitoxin system controlled by a metabolite biosensor which has at least the following features:

1. a first nucleic acid molecule encoding a transcription factor that functions as a biosensor and is capable of binding to the cellular metabolite produced by the cell to form a complex. The complex functions as an "activated transcription complex" in that it is able to interact with a gene promoter and induce expression of its cognate coding sequence; and
2. a second nucleic acid molecule which is operably linked to an inducible promoter and comprises a coding sequence encoding an anti-toxin protein that is cognate to and binds to the toxin, whereby the anti-toxin acts as an antidote to the toxin and permits the survival and growth of the cell; and
3. a third nucleic acid molecule encoding a toxin protein wherein the nucleic acid molecule comprises a coding sequence that is operably linked to a constitutive promoter;

wherein expression of the antitoxin encoded by the second nucleic acid molecule is induced when the transcription factor and the cellular metabolite form a complex. As stated above, this complex, which functions as an "activated transcription complex" is capable of binding to the inducible promoter of the second nucleic acid molecule so as to induce expression of the antitoxin.

The Toxin/Antitoxin (TA) system, as used herein, is a two-component system whose features are used to slow the growth, or eliminate, non-producing micro-organisms that arise in a proliferating cell population during production of a metabolite e.g. during fermentation. The general concept of TA systems is given by the name: one component is a toxin molecule which affects a critical function in a cell, and the second component is an antitoxin that, upon expression in the cell, can cancel the effect of the toxin. The toxin usually causes growth arrest or cell death by impairing functions such as transcription, translation, cell division (replication and cytoskeleton formation), or membrane stability.

Examples of suitable TA systems include the type II TA pair, yefM-yoeB, from *E. coli.* The YoeB protein is a toxin that functions as an mRNA interferase, and binds to the 50S subunit of the ribosome to block translation initiation. The YoeB protein also has endoribonuclease activity without association to the ribosome. The antitoxin, YefM, forms a dimer which binds a single YoeB molecule to form a stable complex, which inactivates the mRNA-degrading action of YoeB. The YefM antitoxin is very sensitive to degradation by the Lon protease, whereby YoeB is then released and causes a growth arrest. Since the antitoxin has a short half-life, the absence of cellular metabolite required for continued expression of the antitoxin rapidly leads to release of the toxin and subsequent growth arrest.

The amino acid sequence of a functional YefM anti-toxin encoded by the coding sequence of the second nucleic acid molecule, has at least 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 99, or 100% sequence identity to SEQ ID No: 2.

The amino acid sequence of a functional YoeB toxin encoded by the coding sequence of the third nucleic acid molecule, has at least 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 99, or 100% sequence identity to SEQ ID No: 3.

Alternative suitable TA pairs include mazF-mazE (SEQ ID No. 36 and 38; encoded by SEQ ID No: 35 and 37 respectively), yafO-yafN (SEQ ID No. 40 and 42; encoded by SEQ ID No: 39 and 41 respectively) and relE-relB (SEQ ID No. 44 and 46; encoded by SEQ ID No: 43 and 45 respectively).

Use of alternative, suitable TA pairs when using the pBAD-TA5 vector requires that the DNA sequences encoding yoeB and yefM in pBAD-TA5 are replaced with the respective toxin and antitoxin encoding sequence above.

The third nucleic acid molecule comprises a constitutive promoter, operably linked to a coding sequence encoding a toxin protein that drives expression of the toxin protein. A suitable promoter is a constitutive promoter, whereby toxin protein is expressed continuously within the cell, for example J23100 having nucleotide sequence SEQ ID No: 48.

The second nucleic acid molecule comprises a coding sequence encoding an anti-toxin protein, operably linked to an inducible promoter that drives expression of the anti-toxin protein. A suitable inducible promoter is one that is activated and induces expression of the cognate coding sequence encoding the antitoxin protein, when the biosensor (a transcription factor) and the cellular metabolite form a complex. More specifically, transcription (and expression of the anti-toxin protein) may be induced on binding of this complex to this inducible promoter.

By way of example only, a suitable inducible promoter includes the p_{BAD} (SEQ ID No: 66), that is inducible by the transcription factor biosensors that bind to the metabolites L-arabinose, and mevalonate, (see examples).

The first nucleic acid molecule encodes a transcription factor that is capable of binding to the cellular metabolite produced by the cell to form a complex. The complex functions as an "activated transcription complex" in that it is able to interact with a gene promoter and induce expression of its cognate coding sequence.

By way of example only, suitable transcription factors encoded by the coding sequence of the first nucleic acid molecule include *AraC* (SEQ ID No: 6), *AraCmev* (SEQ ID No:12) and FadR (SEQ ID No: 68) that function as biosensors by binding to the metabolites L-arabinose, mevalonate and fatty acid / fatty acid acyl-CoA respectively (see examples illustrating biosensor:inducible promoter pairs for a range of metabolites).

The first nucleic acid molecule comprises a promoter operatively linked to a coding sequence encoding the transcription factor. If a eukaryotic host cell is used, the transcription factor should comprise a nuclear localization signal peptide e.g. encoding the protein sequence Pro-Lys-Lys-Lys-Arg-Lys-Val.

A suitable promoter is a constitutive promoter, whereby the biosensor (transcription factor) is expressed continuously within the cell at a level where binding of effector yields responsive gene regulation, for example selected from among the synthetic promoters listed below, for example J23100 having nucleotide sequence of SEQ ID No: 48.

Promoter	Sequence (5'-)	SEQ ID No
J23119	ttgacagctagctcagtcctaggtataatgctagc	47
J23100	ttgacggctagctcagtcctaggtacagtgctagc	48
J23101	tttacagctagctcagtcctaggtattatgctagc	49
J23102	ttgacagctagctcagtcctaggtactgtgctagc	50
J23103	ctgatagctagctcagtcctagggattatgctagc	51
J23104	ttgacagctagctcagtcctaggtattgtgctagc	52
J23105	tttacggctagctcagtcctaggtactatgctagc	53
J23106	tttacggctagctcagtcctaggtatagtgctagc	54
J23107	tttacggctagctcagccctaggtattatgctagc	55
J23108	ctgacagctagctcagtcctaggtataatgctagc	56
J23109	tttacagctagctcagtcctagggactgtgctagc	57
J23110	tttacggctagctcagtcctaggtacaatgctagc	58
J23111	ttgacggctagctcagtcctaggtatagtgctagc	59
J23112	ctgatagctagctcagtcctagggattatgctagc	60
J23113	ctgatggctagctcagtcctagggattatgctagc	61
J23114	tttatggctagctcagtcctaggtacaatgctagc	62
J23115	tttatagctagctcagcccttggtacaatgctagc	63
J23116	ttgacagctagctcagtcctagggactatgctagc	64
J23117	ttgacagctagctcagtcctagggattgtgctagc	65

J23118	ttgacggctagctcagtcctaggtattgtgctagc	89

III: A genetically modified microbial cell comprising a toxin-antitoxin system controlled by a metabolite riboswitch biosensor

According to a second embodiment, the genetically modified microbial cell, which is for use in the intracellular biosynthesis of a given cellular metabolite, is a cell comprising a toxin-antitoxin system controlled by a metabolite biosensor which has at least the following features:

1. a first nucleic acid molecule, and

2. a second nucleic acid molecule comprising a coding sequence,

wherein the first nucleic acid molecule is operably linked to the second nucleic acid molecule upstream if its coding sequence and operably linked downstream of a constitutive promoter; and

wherein the second nucleic acid molecule comprises a coding sequence encoding an anti-toxin protein that is cognate to and binds to the toxin, whereby the anti-toxin acts as an antidote to the toxin and permits the survival and growth of the cell; and

wherein the transcription product of the first nucleic acid molecule is a a riboswitch capable of binding to the cellular metabolite to form a complex;

3. a third nucleic acid molecule encoding a toxin protein wherein the nucleic acid molecule comprises a coding sequence that is operably linked to a constitutive promoter;

wherein expression of the antitoxin encoded by the second nucleic acid molecule is induced when the riboswitch and the cellular metabolite form a complex.

The riboswitch system, as used herein, is a method of regulating expression of the component genes of the synthetic error correction system in the genetically modified micro-organisms of the invention. The regulation takes place at the translational level, and is mediated by mRNA structures which can be formed upstream or downstream of the coding region in the 3'- or 5'-untranslated region (UTR). These riboswitches are RNA structures, which are capable of binding effectors (e.g. small-molecules or metabolites) and modulate transcription or translation of a gene *in cis*. A riboswitch is

composed of two separate domains: an aptamer domain responsible for ligand recognition and binding, and an expression system. These two typically overlap, and the overlap is known as a switching sequence, since it will base pair with either domain depending on the state of the riboswitch. Riboswitches can also modulate gene translation by forming a structure prone to degradation by RNases. Riboswitches can be both ON and OFF switches upon ligand binding, and transcriptional control can be carried out by the formation of terminators and anti-terminators (or anti-anti-terminators) in response to molecule recognition. Translational regulation is also carried out by affecting the availability of the ribosome binding site. The riboswitch two-dimensional structure will either sequester or expose the ribosome binding site upon binding of the small molecule, resulting in the existence of both ON switches (Ribosome binding site exposed upon molecule binding), and in the opposite case, OFF switches, for example by forming a 'road-block' preventing progress of the already bound ribosomal machinery.

By way of example, the nucleotide sequence of a first nucleic acid molecule that is transcribed into a suitable OFF riboswitch is selected from the group consisting of: *btuB* leader (adenosylcobalamin-responsive) [SEQ ID No: 69]; tc3 (tetracycline-responsive) [SEQ ID No: 70]; and ThiMwt (TPP-responsive) [SEQ ID No: 71].
An ON riboswitch, ThiMN15#19, is exemplified in Example 3.

IV: A genetically modified microbial cell comprising a toxin gene controlled by a metabolite OFF-type biosensor

According to a third embodiment, the genetically modified microbial cell, which is for use in the intracellular biosynthesis of a given cellular metabolite, is a cell comprising a toxin system controlled by a metabolite biosensor which has at least the following features:

1. a first nucleic acid molecule wherein the transcription and/or translation of said molecule yields a biosensor capable of binding said cellular metabolite to form a complex, and

2. a second nucleic acid molecule which is operably linked to a constitutive promoter and where the second nucleic acid molecule comprises a coding

sequence encoding a toxin protein that prevents the survival and/or growth of the cell; and

wherein the complex functions to block the transcription and/or translation of the second nucleic acid molecule.

When the first nucleic acid encodes a transcription factor, the complex that is formed on its binding to the cellular metabolite is an "activated transcription complex" that is capable of interacting with the promoter of the second nucleic acid molecule so as to block expression of the toxin.

Alternatively, first nucleic acid molecule is operably linked to the second nucleic acid molecule upstream of its coding sequence of and operably linked downstream of the constitutive promoter, said biosensor obtained on transcription of said first nucleic acid molecule is a riboswitch capable of binding to said metabolite to form a complex, and thereby blocking expression of the toxin.

A suitable "OFF" transcription factor for this third embodiment includes XylR (repressor) biosensor (xylose-responsive) [SEQ ID No: 72] and its cognate responsive promoter [SEQ ID No: 73]); and a suitable "OFF" riboswitch for this variant of the third embodiment includes ThiMwt (TPP-responsive, SEQ ID No: 74)

V: A genetically modified microbial cell comprising an essential gene controlled by a metabolite biosensor

According to a fourth embodiment, the genetically modified microbial cell, which is for use in the intracellular biosynthesis of a given cellular metabolite, is a cell comprising an essential gene whose expression is controlled by a metabolite biosensor and which has at least the following features:

1. a first nucleic acid molecule encoding a transcription factor that functions as a biosensor and is capable of binding to the cellular metabolite produced by the cell to form a complex. The complex functions as an "activated transcription complex" in that it is able to interact with a gene promoter and induce expression of its cognate coding sequence; and

2. a second nucleic acid molecule (also known as an essential gene), which is operably linked to an inducible promoter, and comprises a coding sequence encoding an essential protein, and wherein expression of this essential protein permits the survival and growth of the cell; and

wherein expression of the essential protein encoded by the second nucleic acid molecule is induced when the transcription factor and the cellular metabolite form a complex. As stated above, this complex, which functions as an "activated transcription complex" is capable of binding to the inducible promoter of the second nucleic acid molecule so as to induce expression of the essential protein. Examples of biosensors, encoded by the first nucleic acid molecule, and inducible promoters in the second nucleic acid molecule that control expression of the essential protein, are described in section II.

Essential genes, as used herein, provide an alternative synthetic error correction system, whereby the expression of a single gene product is used to control the fate of the genetically modified micro-organism of the invention. As the name reveals, the products of essential genes include those found necessary for cell growth under a defined set of conditions, as well as genes that become essential for growth of the genetically modified micro-organism of the invention. These conditions include the criteria that most of the necessary components for growth are present, alongside a temperature allowing for optimal growth rates. Characteristic of *E. coli* essential genes is that it is not possible to create viable cells of *E. coli* with knock-outs of these genes under the defined set of conditions.

By way of example only, the following six essential genes are suitable for use as this alternative synthetic error correction system. A characteristic of the following six essential genes is that their over-expression is not lethal for the cell:

murZ [SEQ ID No. 75], encodes an enzyme in the first committed step of peptidoglycan biosynthesis (also known as MurA) [SEQ ID No. 76];

mraY [SEQ ID No. 77], encodes a membrane-bound translocase [SEQ ID No. 78] also termed a UDP-MurNAc-pentapeptide phosphotransferase, located at

the inner membrane of the ER which, alongside the gene product of murG, facilitates lipid II synthesis;

glmM [SEQ ID No. 79], encodes a phosphoglucosamine mutase [SEQ ID No. 80]; The *glmM* gene in *E. coli* encodes a phosphoglucosamine mutase; which catalyzes the isomeric conversion between glucosamine-6-phosphate and glucosamine-1-phosphate. This reaction is one of the first in the biosynthetic pathway leading to the metabolite precursor UDP-Nacetylglucosamine (UDPGlcNAc). This compound forms a branch-point for pathways leading to both peptidoglycan and lipopolysaccaride synthesis, which are both essential cell wall constituents;

murI [SEQ ID No. 81], encodes a glutamase racemase [SEQ ID No. 82], required for synthesis of D-glutamic acid – another essential building block of peptidoglycan, also known as *btuB*;

ribA [SEQ ID No. 83], encodes a GTP cyclohydrolase II [SEQ ID No. 84], which catalyses the first committed step in riboflavin biosynthesis;

adk [SEQ ID No. 85],encodes an adenylate kinase [SEQ ID No. 86], which is an essential part of the nucleotide metabolism and catalyzes phosphorylation of AMP to ADP and dAMP to dATP (and nucleoside diphosphates to their correspond triphosphates).

In one embodiment the essential gene is *glmM*, which is present in a two-gene operon with another essential gene: *folP*. The *folP* gene functions as the essential gene because it is the first gene in this operon, but as seen in the examples, the presence of more than one essential gene in the synthetic error correction system can be used to control the fate of the genetically modified micro-organism of the invention. The *folP* gene [SEQ ID No:87], encodes dihydropteroate synthase [SEQ ID No. 88], which is part of the enzymatic pathway leading to production of tetrahydrofolate (vitamin B9). This compound is essential to normal cell growth, as folic acid cofactors are necessary for production of purines, methionine, thymidine, lysine and pantothenic acid.

VI A cellular metabolite produced by a genetically modified micro-organism of the invention

A cellular metabolite produced by intracellular biosynthesis by the genetically modified micro-organism of the invention may range from a small molecule up to larger products, such as proteins.

A small molecule, by way of example only, is mevalonate, which is a precursor to a diverse group of compounds termed isoprenoids. Isoprenoids cover a group of chemicals with a diverse range of functions, structures, and applications. With over 50.000 known compounds, isoprenoid functions include flavors and perfumes, hormones, mediators of membrane fluidity, and pharmaceuticals. Plants have especially been found to be an incredible reservoir of these diverse secondary metabolites. The existing method of obtaining these interesting molecules (e.g. by plant extraction) is however inefficient. Microbial production of isoprenoids represents a green and feasible alternative to obtaining these compounds.

Further small molecules, may include fatty acid ethyl esters and other biodiesel molecules, which represent another branch of biochemicals that can be produced in microbial cells, ultimately converting glucose or other carbon sources into combustible fuels. Polyunsaturated fatty acids, and many other metabolic products, such as amino acids and organic acids, are already existing or potential fermentation products that can be produced by the microbial cells of the invention and for which synthetic error correction system of the invention can be used, making use of known or adapted transcription or translation regulatory elements. For example, natural riboswitches have been found responsive to various vitamin B family molecules.

The present invention provides a powerful tool for enhancing the productivity of isoprenoid production by microbial cell factories, by eliminating non-producers from continuous fermentation. This is illustrated with respect to mevalonate production in the examples herein.

By way of example, a genetically modified micro-organism for production of mevalonate comprises three genes encoding a biosynthetic pathway for the production of mevalonate from acetyl-CoA, namely: the *E. coli* gene *atoB*, and the two genes *HMGS (ERG13)*, and *tHMGR* from *S. cerevisiae*. The *atoB* gene encodes an acetoacetyl- CoA thiolase, which catalyzes the formation of acetoacetyl-CoA from two molecules of acetyl-CoA. The *HMGS* gene encodes a

HMG-CoA synthase, which forms 3- hydroxy-methylglutayl-CoA (HMG-CoA) by a condensation reaction between acetoacetyl-CoA and another molecule of acetyl-CoA. Finally, HMG-CoA is converted to mevalonate by the tHMGR gene product, which is a truncated version of an *HMGR* gene. The product of HMG-CoA synthase activity is toxic to the cell, and it is the accumulation of HMG-CoA which promotes growth inhibition.

There is a fitness cost related to production of mevalonate, both in the length of the lag phase and the final OD of a producing strain (figure 6A). Both of these traits underline the potential effect of having non-producers appear in a fermentation. As the culture is inoculated, the producing cells will have a long lag phase, leaving room for non-producers to grow and overtake the population. As mutations would most likely appear in relation to growth, this phenomenon would likely be of greater importance when transferring a growing culture into fresh medium, at which point cheater cells had already appeared in the previous cultivation. This would also apply to batch fermentations, where one or more serial pre-cultures are used, as well as for chemostat cultures. As presented in the examples, the TA-correction system is shown to be effective in reducing the relative fitness cost between a producing and non-producing strain by lowering the fitness of the non-producers.

VII Methods for producing a cellular metabolite using the genetically modified micro-organism of the invention

According to a further embodiment, the invention provides a method for producing a biosynthetic metabolite comprising the steps of: a) providing a genetically modified microbial cell as defined above in sections I - IV, b) introducing the genetically modified microbial cell into a cultivation medium comprising a substrate for production of said metabolite, and c) recovering metabolite produced by said culture, wherein a lack of metabolite production in said genetically modified microbial cell or progeny cell thereof attenuates multiplication of said cell as compared to a non-genetically modified parent cell from which said modified microbial cell was derived.

In step b) it is contemplated that the cell in culture medium are cultivated under continuous; fed-batch or batch culture; and that the cells undergo at

least 10, 15, 20, 25, 30, 35, 40, 45 or 50 generations of cell multiplication. The period of cultivation will depend on the micro-organism cultivated; but where the micro-organism is a bacterial cell, the accumulated period of cultivation is typically at least 2, 4, 6, 8, 10, 12, 14 , 16, 18 or 20 days. In the context of the present invention the term "accumulated period of cultivation" is to be understood to include the cultivation of a pre-seed culture; further cultivation after subsequent inoculation of the pre-seed culture into a larger fermenter, and optionally cultivation after subsequent inoculation of the previous culture into an even larger fermenter. Production of a metabolite using the genetically modified microbial cell of the invention wherein the production of the metabolite has a high fitness cost of production, such as ≥ 5 %, ≥10%, ≥15%, ≥20%, and ≥25%.

VIII Micro-organisms for the intracellular biosynthesis of a cellular metabolite

The micro-organism for the intracellular biosynthesis of a cellular metabolite according to the invention, may be a bacterium, a non-exhaustive list of suitable bacteria is given as follows: a species belonging to the genus *Bacillus*, a species belonging to the genus *Escherichia*, a species belonging to the genus *Lactobacillus*, a species belonging to the genus *Lactococcus*, a species belonging to the genus *Corynebacterium*, a species belonging to the genus *Acetobacter*, a species belonging to the genus *Acinetobacter*, a species belonging to the genus *Pseudomonas*; a species belonging to the genus *Proprionibacterium,* and a species belonging to the genus *Bifidobacterium*.

Alternatively, the micro-organism according to the invention may be a yeast belonging to the genus of Saccharomyces, e.g. *S. cerevisiae, S. kluyveri, S. bayanus, S. exiguus, S. sevazzi, S. uvarum*; a yeast belonging to the genus *Kluyveromyces*, e.g. *K. lactis K. marxianus var. marxianus, K. thermotolerans*; a yeast belonging to the genus Candida, e.g. *C. utilis C. tropicalis, C. albicans, C. lipolytica, C. versatilis*; a yeast belonging to the genus Pichia, e.g. *P. stipidis, P. pastoris, P. sorbitophila*, or other yeast genera, e.g. *Cryptococcus, Debaromyces, Hansenula, Yarrowia, Zygosaccharomyces* or *Schizosaccharomyces*. Alternatively, the micro-

organisms may be a filamentous fungus belonging to the genus of *Penicillium, Rhizopus, Fusarium, Fusidium, Gibberella, Mucor, Mortierella, Trichoderma Thermomyces, Streptomyces* and *Aspergillus.* More specifically, the micro-organism may be *Fusarium Oxysporum, A. niger, A. awamori, A. oryzae,* and *A. nidulans*.

The preferred micro-organisms of the invention may be *S. cerevisiae, E. coli, L. lactis* or *L. plantarum. Bacillus subtilis, B. licheniformis, Trichoderma resei, Aspergillus niger, Aspergillus oryzae, Yarrowia lypolytica,* and *Pichia pastoris.*

IX Methods for producing a micro-organism of the invention

Integration and self-replicating vectors, suitable for cloning and introducing a first, second, third or additional nucleic acid molecules into a micro-organism for the intracellular biosynthesis of a cellular metabolite, are commercially available and known to those skilled in the art (see, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989). Cells of a micro-organism are genetically engineered by the introduction into the cells of heterologous DNA (RNA). Heterologous expression of genes encoding one or more polypeptide of the correction system in a micro-organism of the invention is demonstrated in the Examples.

A first, second, third or additional nucleic acid molecule(s) according to the invention, can be introduced into a cell or cells on plasmids or optionally integrated into the host cell genome using methods and techniques that are standard in the art. For example, nucleic acid molecules can be introduced by standard protocols such as transformation including chemical transformation and electroporation, transduction, particle bombardment, etc.

Examples

Example 1. TA systems

1.1 L-arabinose addiction coupled to a Type II TA system is shown to control bacterial cell growth

The yefM-yoeB TA pair comprises two genes, where the *yoeB* gene encodes a toxin, and the *yefM* gene encodes its cognate antitoxin (figure 2). The YoeB

toxin has two properties that serve to block cell growth: 1) acting as an mRNA interferase, and binding to the 50S subunit of the ribosome to block translation initiation; 2) acting as an endoribonuclease, and degrading mRNA independent of the ribosome. When a cognate YefM dimer is expressed, this binds a single YoeB molecule to form a stable complex and thereby prevents the growth inhibitory properties of the toxin. The half-life of the YefM antitoxin is short, since it is rapidly proteolytically degraded (Lon protease). Accordingly, a failure to express the YefM antitoxin leads to release of the YoeB toxin, which in turn arrests cell growth.

The use of the yefM-yoeB TA pair system to control the growth of bacteria based on L-arabinose addiction was demonstrated in *E. coli*, as follows.

The yefM-yoeB TA pair was cloned into a plasmid (pBAD-TA5: *ara*C, p_{BAD}-*yefM*, p_{Lac}-*yoeB*, *ampR*) (Table 1) employing standard PCR cloning protocols (known in the art) and the plasmid was transformed into the host stain *E. coli* XL-1 with the genotype: *recA1endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F'proAB lacIqZM15 Tn10* (*TetR*)]. Electroporation recovery of transformed cells was carried out in SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, and 20 mM glucose).

Transformed *E. coli* cultures were grown in 2xYT medium (10 g/L yeast extract, 16 g/L tryptone, 5 g/L NaCl). Antibiotics were added to select for maintenance of plasmids in strains transformed with these, according to their respective antibiotic resistance gene. Antibiotic concentrations when added were as follows: ampicillin 100µg/mL, chloramphenicol 30µg/mL, spectinomycin 50µg/mL, kanamycin 50µg/mL.

The expression of the *yoeB* gene in the yefM-yoeB TA pair construct (figure 2) was placed under the control of the Lac promoter, which is inducible by isopropyl β-D-1-thiogalactopyranoside (IPTG). Exposure of the cells to increasing concentrations of IPTG induced sufficient expression of the YoeB toxin to prevent growth, when the cells were grown in the absence of any source of L-arabinose (figure 3A). The expression of the yefM gene is placed under the control of the p_{BAD} promoter, which is inducible by L-arabinose.

The regulation of the p_{BAD} promoter by AraC is dual. In the absence of L-arabinose, the AraC protein binds to operator sites within p_{BAD} effectively repressing transcription. Upon binding of L-arabinose, the complex changes to another DNA-binding conformation leading to activation of p_{BAD} and induction of transcription of a cognate gene.

While cell growth was strongly inhibited by addition of ≥0.1mM IPTG of the toxin inducer; the co-addition of 0.1% L-arabinose was sufficient to restore wild-type growth in the presence of IPTG at concentrations up to 0.5mM (figure 3B).

These data demonstrate that the TA system has a wide dynamic range, with gradual response in respect of growth inhibition. Thus a small change in the level of toxin expression relative to antitoxin expression, does not give rise to a dramatic change in cell growth rates, but is still provides sufficiently strong growth control to prevent growth as well as allow normal growth at extremes in the concentration of the inducer L-arabinose.

Table 1 listing the genetic features of the L-arabinose TA addicted strain based on the *E. coli* XL1 parent strain.

Table 1

Plasmid/chromosome	**Gene**	**SEQ ID No:**	**Protein**	**SEQ ID No**
pBAD-TA5	*ara*C*	SEQ ID No: 1		
	p_{BAD}-*yefM*		YefM	SEQ ID No: 2
	p_{Lac}-*yoeB*		YoeB	SEQ ID No: 3
	ampR		Beta-lactamase	SEQ ID No: 4
	araC	SEQ ID No: 5	araC	SEQ ID No: 6

**araC* gene [SEQ ID No: 5] encoding araC biosensor protein [SEQ ID No: 6] is located on the complementary strand to SEQ ID No: 1 at nucleotide positions: 96 – 974. The p_{BAD} promoter is located a position 1246-1318 in SEQ ID No: 1.

Table 2 listing the genetic features of the mevalonate TA addicted strain m14 based on the *E. coli* XL1 parent strain. The chromosomally located sequence

is an excerpt of the genome sequence that fully comprises the engineered features.

Table 2

Plasmid/chromosome	Gene	SEQ ID No:	Protein	SEQ ID No
pBAM-TA5	*ara*Cmev*	SEQ ID No: 7		
	p_{BAD}-*yefM*		YefM	SEQ ID No: 8
	p_{Lac}-*yoeB*		YoeB	SEQ ID No: 9
	ampR		Beta-lactamase	SEQ ID No: 10
	*ara*Cmev	SEQ ID No: 11	araCmev	SEQ ID No: 12
chromosome	*yefM- FRT-kanR-FRT-yoeB***	SEQ ID No: 13	Neomycin phosphotransferase II	SEQ ID No: 14

**araCmev* gene [SEQ ID No: 11] encoding araCmev biosensor protein [SEQ ID No: 12] is located on the complementary strand to SEQ ID No: 7 at nucleotide positions: 96 - 974;

** (1^{st}) "FRT site" is located on complementary stand to SEQ ID No 13 at nucleotide positions: 247 -281; (2^{nd}) "FRT site" is located on complementary stand to SEQ ID No 13 at nucleotide positions: 1469 - 1503.

The p_{BAD} promoter is located a position 1241-1318 in SEQ ID No: 7.

In order to enhance to the stability of this plasmid based TA correction system, the native genomic copy of the *yefM-yoeB* gene pair was knocked-out. This was achieved using a DNA fragment comprising a FRT-kana-FRT resistance cassette fused to flanking sequences homologous to circa 200bp flanking sequences homologous to each side of the junction between the chromosomal *yefM-yoeB* gene pair. Site specific integration of the FRT-kana-FRT resistance cassette into the native *yefM-yoeB* gene pair and functional knock-out was achieved by means of lambda-red-mediated recombineering.

1.2 Mevalonate addiction coupled to a Type II TA system is shown to control bacterial cell growth

Mevalonate is one of the early precursors in the biosynthesis of a diverse group of compounds termed isoprenoids with a range of applications. The production of mevalonate in micro-organisms is seen to place a metabolic strain on the producing cells, meaning there is a fitness cost, and where the

appearance of non-producing cells compromises the economics of mevalonate production. A Type II TA system coupled to mevalonate addiction according to the invention provides a method for slowing the growth or eliminating cells that are non-producers.

In order to control bacterial cell growth by mevalonate addiction, a mevalonate biosensor was introduced into the yefM-yoeB TA pair plasmid, pBAD-TA5 (Table 2), in place of the L-arabinose biosensor. The mevalonate-responsive biosensor was derived from the AraC sensor by introducing 4 point mutations (figure 4). In the absence of expression of the yefM antitoxin, cell growth was inhibited by the yoeB gene expressed under the control of the p_{LAC} promoter. Addition of the inducer, IPTG, was not required, since the baseline activity of the Lac promoter was sufficient to drive expression of toxin at cell growth inhibitory levels; likely due to the leakiness of the Lac promoter.

Addition of mevalonate (37 mM) was sufficient to bind to the mevalonate biosensor and induce the cognate p_{BAD} promoter to express the YefM antitoxin at levels sufficient to allow an increase in cell growth when compared to cell cultures devoid of mevalonate.

1.3 Engineering a bacterial strain addicted to internal small molecule production

Mevalonate-addicted *E. coli* strains (as described in 1.2) were additionally engineered to produce mevalonate. Strains were transformed with a plasmid comprising genes encoding one of three alternative biosynthetic pathways for the conversion of endogenous acetyl CoA to mevalonate (Figure 5). The plasmid, pMevTC, comprises an operon with the *E. coli* gene *AtoB*, encoding an acetyl-CoA acetyltransferase; an *S. cerevisiae ERG13* gene encoding an HMG-CoA synthase, and *tHMGR*, a truncated version of *S. cerevisiae HMGR*, encoding an HMG-CoA reductase. This plasmid directs intermediate levels of mevalonate production in a host strain (1-2g/L).

The plasmid, pMEV7C, comprises the *E. coli* gene *AtoB*; a *Lactococcus lactis* gene *mvaS* encoding an HMG-CoA synthase, and a *Lactococcus lactis mvaE*

gene encoding a HMG-CoA reductase. This plasmid directs high level mevalonate production in a host strain (≤14.6g/L in batch fermentation).

A negative control pathway pMevT5c features a point mutation in the *ERG13* gene, rendering the strain incapable of producing mevalonate, but otherwise it maintains the same promoter, enzymatic pathway reactions, antibiotic selection gene and plasmid origin of replication. The pathway operon promoter in both plasmids pMEV7C and pMevT5C was the synthetic promoter J23100 [SEQ ID No: 48].

Table 3 listing the genetic features of the mevalonate TA addicted strain *m29* with internal mevalonate biosynthesis, based on the *E. coli* XL1 parent strain.

Table 3

Plasmid/ chromosome	**Gene**	**SEQ ID No:**	**Protein**	**SEQ ID No**
pBAM-TA5	*araCmev**	SEQ ID No: 7		
	p_{BAD}-*yefM*		YefM	SEQ ID No: 8
	p_{Lac}-*yoeB*		YoeB	SEQ ID No: 9
	ampR		Beta-lactamase	SEQ ID No: 10
	araCmev	SEQ ID No: 11	araCmev	SEQ ID No: 12
chromosome	*yefM- FRT-kanR-FRT-yoeB***	SEQ ID No: 13	Neomycin phosphotransferase II	SEQ ID No: 14
pMEV7C	*atoB*	SEQ ID No: 15	acetyl-CoA acetyltransferase	SEQ ID No: 16
	mvaS		HMG-CoA synthase	SEQ ID No: 17
	mvaE		HMG-CoA reductase	SEQ ID No: 18
	*camR****			
	camR	SEQ ID No: 19	Chloramphenicol acetyltransferase	SEQ ID No: 20

**araCmev* gene [SEQ ID No: 11] encoding araCmev biosensor protein [SEQ ID No: 12] is located on the complementary strand to SEQ ID No: 7 at nucleotide positions: 96 - 974;

**(1st) "FRT site" is located on complementary stand to SEQ ID No 13 at nucleotide positions: 247 -281; (2nd) "FRT site" is located on complementary stand to SEQ ID No 13 at nucleotide positions: 1469 - 1503.

***camR* gene [SEQ ID No: 19] encoding chloramphenicol acetyltransferase [SEQ ID No: 20] is located on the complementary strand to SEQ ID No: 15 at nucleotide positions 4745 – 5404. The p_{BAD} promoter is located a position 1241-1318 in SEQ ID No: 7.

Table 4 listing the genetic features of the mevalonate TA addicted strain *m16* without internal mevalonate biosynthesis, based on the *E. coli* XL1 parent strain.

Table 4

Plasmid/ chromosome	**Gene**	**SEQ ID No:**	**Protein**	**SEQ ID No**
pBAM-TA5	*araCmev**	SEQ ID No: 7		
	p_{BAD}-*yefM*		YefM	SEQ ID No: 8
	p_{Lac}-*yoeB*		YoeB	SEQ ID No: 9
	ampR		Beta-lactamase	SEQ ID No: 10
	araCmev	SEQ ID No: 11	araCmev	SEQ ID No: 12
chromosome	*yefM- FRT-kanR-FRT-yoeB***	SEQ ID No: 13	Neomycin phosphotransferase II	SEQ ID No: 14
pMevT5C	*atoB*	SEQ ID No: 21	acetyl-CoA acetyltransferase	SEQ ID No: 22
	Mutant ERG13		Non-functional HMG-CoA synthase	SEQ ID No: 23
	tHMGR		Truncated HMG-CoA reductase	SEQ ID No: 24
	*camR****			
	camR	SEQ ID No: 19	Chloramphenicol acetyltransferase	SEQ ID No: 20

**araCmev* gene [SEQ ID No: 11] encoding araCmev biosensor protein [SEQ ID No: 12] is located on the complementary strand to SEQ ID No: 7 at nucleotide positions: 96 - 974;

**(1^{st}) "FRT site" located on complementary stand to SEQ ID No 13 at nucleotide positions: 247 - 281; (2^{nd}) "FRT site" located on complementary stand to SEQ ID No 13 at nucleotide positions: 1469 - 1503.

***camR* gene [SEQ ID No: 19] encoding chloramphenicol acetyltransferase [SEQ ID No: 20] is located on the complementary strand to SEQ ID No: 21 at nucleotide positions 5293 - 5952.

1.4 Mevalonate-addiction coupled to a TA system cancels the fitness advantage of non-mevalonate producing cells.

Mevalonate production has a fitness cost for a bacterial cell. This is shown by the retarded growth of an E. coli strain, comprising the plasmid (pMEV7C) and producing 'high' level mevalonate, as compared to an E. coli strain, comprising the control plasmid (pMevT5c) and producing no mevalonate, but still having the metabolic burden of expressing the enzymes of the inactivated mevalonate biosynthesis pathway (figure 6A). The product of HGMS activity is known to be toxic to the cell, and it is the accumulation of HMG-CoA which promotes growth inhibition.

Co-expression of the mevalonate-addiction TA system (pBAM-TA5), despite a longer lag phase in these cells, was effective in countering the fitness advantage of non-producing cells (figure 6B). Survival and growth of cells expressing the TA system (pBAM-TA5), depends on a sufficient level of mevalonate to induce expression of antitoxin. The rate of accumulation of internally synthesized mevalonate to levels sufficient to induce antitoxin production may be a factor contributing to the observed lag phase in producing cells that express mevalonate-addiction TA system.

1.5 Use of the TA system to increase small-molecule production by bacterial cultures comprising non-producing cells

The ability of the TA system to favor survival and growth of productive cells (i.e. cells producing the addiction molecule, mevalonate), was demonstrated by co-culturing mevalonate-producing cells, comprising the pMEV7C plasmid, and non-producing strains, comprising the pMevT5C plasmid. The ratios of non-producing:producing cells tested were: 0:100, 75:25 and 90:10. The total mevalonate production of the 3 co-cultured strains was tested with and without co-expression of the mevalonate-addiction TA system, encoded on the plasmid pBAM-TA5.

Previous experiments with strains possessing the mevalonate pathway had established a fitness cost of harboring a functional production plasmid

(pMEV7c) versus a non-functional production plasmid (pMevT5c) to be a reduction in growth rate of approximately 20 % (Figure 6A). Accordingly, the growth of producing cells was by default expected to be disfavored compared to the non-producing cells (i.e. without any synthetic correction system).

The same cell inoculum mixes were cultured with the producing and non-producing strains, but both also harbored the correction plasmid (pBAM-TA5). This addition of the correction system was shown to significantly enhance mevalonate production in the co-cultures comprising non-producing cells (figure 7).

Example 2 Essential genes

2.1 L-arabinose addiction by regulated expression of an essential gene is shown to control bacterial cell growth

The essential genes, used to control bacterial growth, are the *E. coli* genes *folP* and *glmM*, which are comprised together within a two-gene operon. The gene *glmM* encodes a phosphoglucosamine mutase; and the gene *folP* encodes a dihydropteroate synthase, which is part of the enzymatic pathway leading tetrahydrofolate (vitamin B9) synthesis, which is essential for normal cell growth.

The native single genomic copy of the essential gene operon *folP, glmM* in *E. coli* host strain XL1, was modified to allow its transcriptional control by the L-arabinose/mevalonate-responsive promoter p_{BAD}. Replacement of the native promoter by the p_{BAD} promoter was engineered by introducing a knockout fragment containing a kanamycin resistance cassette and the p_{BAD} promoter, as illustrated in the cartoon in figure 8. An L-arabinose biosensor (*AraC*) (Table 1) was introduced into the modified host strain on the plasmid pBAD18-cam. The modified and transformed *E. coli* strains were grown in 2xYT medium (10 g/L yeast extract, 16 g/L tryptone, 5 g/L NaCl). Antibiotics were added to select for maintenance of plasmids in transformed strains, according to their respective antibiotic resistance gene. Antibiotic concentrations when added were as follows: ampicillin 100µg/mL, chloramphenicol 30µg/mL, spectinomycin 50µg/mL, kanamycin 50µg/mL.

Table 5 lists the genetic features of the essential gene-based mevalonate-addicted strain *e3.9*, based on an *E. coli* XL1 parent strain. The chromosomally located sequence is an excerpt of the genome sequence that fully comprises the engineered features.

Table 5

Plasmid/chromosome	**Gene**	**SEQ ID No:**	**Protein**	**SEQ ID No**
chromosome	*(FRT-kanR-FRT)-p_{BAD}-folP**	SEQ ID No: 25	Dihydropteroate synthase	SEQ ID No: 26
	*kanR***	SEQ ID No: 27	Neomycin phosphotransferase II	SEQ ID No: 28
pBAMspec	*araCmev****	SEQ ID No: 29		
	specR		spectinomycin resistance protein	SEQ ID No: 30
pMEV7C	*atoB*	SEQ ID No: 15	acetyl-CoA acetyltransferase	SEQ ID No: 16
	mvaS		HMG-CoA synthase	SEQ ID No: 17
	mvaE		HMG-CoA reductase	SEQ ID No: 18
	*camR*****			

*The native promoter of the chromosomal *folP – glmM* operon is replaced by the p_{BAD} promoter via the (*FRT-KanR-FRT*)-p_{BAD}-*folP* cassette [SEQ ID No. 25]; which comprises the pBAD promoter at position 1907 – 1979 and a portion of the *folP* gene. The nucleotide sequence of the *folP* gene in the *e3.9* strain is SEQ ID No: 87.

***kanR* gene [SEQ ID No: 27] encoding Neomycin phosphotransferase II [SEQ ID No: 28] is located on the complementary strand to SEQ ID No: 25 at nucleotide positions 389 - 1183.

****araCmev* gene [SEQ ID No: 11] encoding araCmev biosensor protein [SEQ ID No: 12] is located on the complementary strand to SEQ ID No: 29 at nucleotide positions: 96 – 974.

*****camR* gene [SEQ ID No: 19] encoding chloramphenicol acetyltransferase [SEQ ID No: 20] is located on the complementary strand to SEQ ID No: 21 at nucleotide positions 4745 - 5404.

Table 6 listing the genetic features of the essential gene-based L-arabinose-addicted strain *e3.5*, based on an *E. coli* XL1 parent strain. The chromosomally located sequence is an excerpt of the genome sequence that fully comprises the engineered features.

Table 6

Plasmid/chromosome	Gene	SEQ ID No:	Protein	SEQ ID No
chromosome	*(FRT-kanR**-FRT)-p_{BAD}-folP**	SEQ ID No: 25	Dihydropteroate synthase	SEQ ID No: 26
pBAD18-cam***	*araC*		AraC	
	cam		Chloramphenicol acetyltransferase	

*The native promoter of the chromosomal *folp – glmM* operon is replaced by the p_{BAD} promoter via the (*FRT-KanR-FRT*)-p_{BAD}-*folP* cassette [SEQ ID No. 25]; which comprises the pBAD promoter at position 1907 – 1979 and a portion of the *folP* gene. The nucleotide sequence of the *folp* gene in the *e3.5* strain is SEQ ID No: 87.

**kanR* gene [SEQ ID No: 27] encoding Neomycin phosphotransferase II [SEQ ID No: 28] is located on the complementary strand to SEQ ID No: 25 at nucleotide positions 389 - 1183.

*** Guzman et al, 1995

Table 7 listing the genetic features of the *pe1* control strain producing mevalonate without the addiction system, based on an *E. coli* XL1 parent strain.

Table 7

Plasmid/chromosome	Gene	SEQ ID No:	Protein	SEQ ID No
pBAM18-spec	*araCmev**	SEQ ID No: 29	AraCmev	SEQ ID No: 12
	specR		aminoglycoside nucleotidyltransferase	SEQ ID No: 30
pMEV7C	*atoB*	SEQ ID No: 15	acetyl-CoA acetyltransferase	SEQ ID No: 16
	mvaS		HMG-CoA synthase	SEQ ID No: 17
	mvaE		HMG-CoA reductase	SEQ ID No: 18
	*camR***		chloramphenicol acetyltransferase	SEQ ID No: 20

* *araCmev* gene [SEQ ID No: 11] encoding araCmev biosensor protein [SEQ ID No: 12] is located on the complementary strand to SEQ ID No: 29 at nucleotide positions: 96 - 974;

**camR* gene [SEQ ID No: 19] encoding chloramphenicol acetyltransferase [SEQ ID No: 20] is

located on the complementary strand to SEQ ID No: 21 at nucleotide positions 4745 – 5404; *specR* = spectomycin resistance protein (e.g. aminoglycoside nucleotidyltransferase).

Since the expression levels of essential genes is important for optimizing their use for growth control, a micro-library of four different ribosome binding sites (created by a single random base pair in one of the primers covering the RBS in front of the p_{BAD} promoter), having different (RBS) strengths, were tested and one RBS was found to give a particularly good conditional growth, strictly in response to providing L-arabinose in the growth medium (Figure 9). When cells, comprising the essential gene operon (*folP/glmM*) driven by the L-arabinose responsive p_{BAD} promoter, were cultured in 2xYT growth medium supplemented with 0.25 % L-arabinose at 37 °C, their growth was exponential, whereas cells with supplemented with only 0.0025% and 0% L-arabinose completely failed to grow over a 12-hour time course.

2.2 Use of essential genes to enhance small molecule production in bacteria.

An *E. coli* strain comprising the essential gene operon *folP-glmM*, whose expression was regulated by a mevalonate-addiction, was engineered in order to demonstrate its use for the regulation of mevalonate-dependent cell growth.

The native single genomic copy of the essential gene operon *folP-glmM* in *E. coli* host strain XL1, was modified to allow its transcriptional control by the L-arabinose/mevalonate-responsive promoter p_{BAD} (figure 8). A mevalonate biosensor (*AraCmev*) (Table 3) was introduced into the modified host strain on the plasmid pBAM18-spec.

In order to demonstrate that growth and mevalonate production by the "essential gene" regulated *E. coli* strain was dependent on internal mevalonate production the cells were also transformed with a plasmid carrying the genes encoding the mevalonate biosynthetic pathway (pMEV7C (Table 2) or genes encoding the inactivated the mevalonate biosynthetic pathway (pMevT5C). Growth of these *E. coli* strains of producing strains (having a functional mevalonate pathway) was exponential, while growth of non-producing strains (having an inactivated mevalonate pathway) was strongly inhibited (figure 10).

2.3 Triacetic acid lactone addiction by regulated expression of an essential gene is shown to control bacterial cell growth

The native single genomic copy of the essential gene operon *folP-glmM* in the *E. coli* XL1 host strain was modified to allow its transcriptional control by the triacetic acid lactone-responsive promoter p_{BAD}. First, a gene encoding a triacetic acid lactone biosensor (AraCtal (Tang et al., 2013)) was introduced into the XL1 host strain on the plasmid pBALspec (Table 8). Then, replacement of the native *folP-glmM* promoter in this host strain by the p_{BAD} promoter was engineered by means of lambda red-mediated recombineering. Recombineering was performed using well-described methods utilizing the pKD46 plasmid (Datsenko and Wanner, 2000), but instead of gene disruption, a specific set of knock-out DNA fragments (produced by PCR) was used to only replace the native essential *folP-glmM* operon promoter and *folP* ribosomal binding site (RBS). This set of knockout DNA fragments each contained a kanamycin resistance cassette and the p_{BAD} promoter, as illustrated in the cartoon in figure 8. The nucleotide sequence of the set of gene knockout DNA fragments differed only with respect to the redundant nucleotide ("N", wherein "N" is any of A, T, G or C) in the RBS sequence (ACTTGC) for *folP-glmM,* which generated a four-membered library of *folP* translational strengths. This library allowed selection of a translational strength for *folP* that resulted in control of normal cell growth in response to transcriptional regulation by the AraCtal biosensor.

Table 8: TAL responsive e3.16#5 clone

Plasmid/chromosome	**Gene**	**SEQ ID No:**	**Protein**	**SEQ ID No**
chromosome	*(FRT-kanR-FRT)-p_{BAD}-folP**	SEQ ID No: 91*	Dihydropteroate synthase	SEQ ID No: 26
	*kanR***	SEQ ID No: 27	Neomycin phosphotransferase II	SEQ ID No: 28
pBALspec	*AraCtal* ***	SEQ ID No: 93	Triacetic acid lactone sensor protein	SEQ ID No:96

	specR		spectinomycin resistance protein	SEQ ID No: 94

*The native promoter of the chromosomal *folP – glmM* operon is replaced by the p_{BAD} promoter via the (*FRT-KanR-FRT)-p_{BAD}-folp* cassette [SEQ ID No.91]; which comprises a p_{BAD} promoter at position 1665 – 1990, a RBS at position 1980-1985, having the nucleotide sequence ACTTGC; and a portion of the *folP* gene. The nucleotide sequence of the complete *folP* gene in the *e3.16#5* strain is SEQ ID No: 87.

**kanR* gene [SEQ ID No: 27] encoding Neomycin phosphotransferase II [SEQ ID No: 28] is located on the complementary strand to SEQ ID No: 91 at nucleotide positions 389 - 1183.

***araCtal* gene [SEQ ID No: 95] encoding araCtal biosensor protein [SEQ ID No: 96] is located on the complementary strand to SEQ ID No: 93 at nucleotide positions: 96 – 1025.

****specR* gene encoding spectinomycin resistance protein [SEQ ID No: 94] is located at nucleotide positions 1945-2916 of SEQ ID No: 93.

For recombineering, as performed in standard pKD46 protocols, the target strain was first transformed with pKD46, and a single colony of this strain was then cultured in a 25 mL 2xYT culture at 30 degrees C and 250 rpm horizontal shaking. When the culture reached OD_{600} = 0.1, the lambda Red system of pKD46 was induced by addition of 0.2 vol% L-arabinose, and then further cultured to grow to OD_{600} = 0.4. The cells from the culture were then transformed with 300-500 ng knock-out DNA fragments by standard high-efficiency electroporation; the electroporated cells were then cultured in SOC medium (20 g/L tryptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) at 37 degrees for at least 1-2 hours to recover and simultaneously cure the cells of the pKD46 plasmid. Following recovery, candidate recombineered cells were selected for by plating on LB agar plates supplemented with 10 mM triacetic acid lactone and spectinomycin for maintenance of pBALspec and kanamycin for selection of inserted knockout fragment.

A non-addicted control *E. coli* strain (e3.16CON), also equipped with spectinomycin and kanamycin resistance genes, but with wildtype control of *folP-glmM* expression (Table 9), was constructed in order to test the toxicity of triacetic acid lactone.

Table 9 Non-addicted control strain e3.16CON

Plasmid/chromosome	**Gene**	**SEQ ID No:**	**Protein**	**SEQ ID No**

chromosome	*kanR*	SEQ ID No: 27	Neomycin phosphotransferase II	SEQ ID No: 28
pBALspec	*AraCtaI* ***	SEQ ID No: 93	Triacetic acid lactone sensor protein	SEQ ID No:96
	specR		Spectinomycin resistance protein	SEQ ID No: 98

****araCtaI* gene [SEQ ID No: 95] encoding araCtaI biosensor protein [SEQ ID No: 96] is located on the complementary strand to SEQ ID No: 93 at nucleotide positions: 96 – 1025.

The non-addicted control *E. coli* strain (e3.16CON), and a selected addicted strain (e3.16#5) were then tested and compared for triacetic acid lactone-dependent growth by cultivation in a microtiter plate reader in liquid 2xYT medium supplemented with spectinomycin and kanamycin and a three-step gradient of triacetic acid lactone (0, 2 and 20 mM). As seen in figure 11, the control strain showed a relative reduction in growth rate as a result of supplementation with triacetic acid lactone, indicating that triacetic acid lactone is toxic for growth. The observed growth depression was greatest at a concentration of 20mM triacetic acid lactone.

The addicted strain e3.16#5 was able to grow in the absence of triacetic acid lactone, indicating that under non-inducing conditions the basal expression of the essential *folP-glmM* operon in this strain was sufficient to support growth. Cell growth was however significantly reduced to a level matching the growth of the control strain under toxic condition of 20 mM triacetic acid lactone (Figure 11 and Figure 12). In contrast to the control strain, the growth rate of the addicted strain e3.16#5 was enhanced by the presence of 2 mM triacetic acid lactone, and the final cell yield was increased by the presence of both 2mM and 20mM triacetic acid lactone (Figure 12). The dependence of the addicted strain on a supply of triacetic acid lactone for maximal growth (figure 12), confirms that the triacetic acid lactone biosensor is able to induce the p_{BAD} promoter and the expression of dihydropteroate synthase, in the pathway leading tetrahydrofolate (vitamin B9) synthesis, which is essential for normal cell growth.

2.4 . Salicylic acid addiction by regulated expression of an essential gene is shown to control bacterial cell growth

The native single genomic copy of the essential gene operon *folP-glmM* in the *E. coli* XL1 host strain was modified to allow its transcriptional control by the salicylic acid-responsive promoter p_{SAL} [SEQ ID No:118]. First, a gene encoding a salicylic acid biosensor (*Pseudomonas putida nahR_Asn169* (Cebolla et al., 1997)) was introduced into the XL1 host strain on the plasmid pBANspec (Table 10). Then, replacement of the native *folP-glmM* promoter by the p_{SAL} promoter was engineered by means of lambda red-mediated recombineering.

Table 10 Salicylic acid responsive e3.18#1 clone

Plasmid/chromosome	Gene	SEQ ID No:	Protein	SEQ ID No
chromosome	*(FRT-kanR-FRT)-p_{SAL}-folP**	SEQ ID No: 101	Dihydropteroate synthase	SEQ ID No:102
	*kanR***	SEQ ID No: 27	Neomycin phosphotransferase II	SEQ ID No: 28
pBANspec	*NahRAsn169* ***	SEQ ID No: 97	Salicylic acid biosensor protein	SEQ ID No:100
	specR		Spectinomycin resistance protein	SEQ ID No: 98

*The native promoter of the chromosomal *folP – glmM* operon is replaced by the p_{SAL} promoter via the *(FRT-KanR-FRT)-p_{BAD}-folP* cassette [SEQ ID No.101]; which comprises p_{SAL} promoter at position 1794 – 1929; a RBS at position 1930-1935, having the nucleotide sequence ACTTGT; and a portion of the *folP* gene (to direct homologous recombination). The nucleotide sequence of the complete *folP* gene in the *e3.18#1* strain is SEQ ID No: 87.

***kanR* gene [SEQ ID No: 27] encoding Neomycin phosphotransferase II [SEQ ID No: 28] is located on the complementary strand to SEQ ID No: 91 at nucleotide positions 389 - 1183. ****nahRAsn169* gene [SEQ ID No: 99] encoding salicylic acid biosensor protein [SEQ ID No: 100] is located on the complementary strand to SEQ ID No: 97 at nucleotide positions: 102 - 1004.

Recombineering was performed as described in section 2.3 using well-described methods utilizing the pKD46 plasmid (Datsenko and Wanner, 2000). Instead of gene disruption, a specific set of knock-out DNA fragments was used to only replace the native essential *folP-glmM* operon promoter and *folP* RBS. This set of knockout DNA fragments contained a kanamycin resistance cassette and the p_{SAL} promoter, as illustrated in the cartoon in figure 8, as well as a redundant nucleotide ("N", wherein "N" is any of A, T , G or C) in the RBS for *folP-glmM* to generate a four-membered library of *folP* translational strengths. This library allows selection of a translational strength

for *folP* that suits the transcriptional regulation by the biosensor. Finally, candidate recombineered cells were selected for by plating on LB agar plates supplemented with 5 mM salicylic acid and spectinomycin for maintenance of pBANspec; and kanamycin for selection of inserted knockout fragment. A control host strain e3.18CON (Table 11) also equipped with spectinomycin and kanamycin resistance genes, but with wild-type control of *folP-glmM* expression) was used to in order to test the toxicity of Salicylic acid.

Table 11 Non-addicted control strain e3.18CON

Plasmid/chromosome	**Gene**	**SEQ ID No:**	**Protein**	**SEQ ID No**
chromosome	*kanR*	SEQ ID No: 27	Neomycin phosphotransferase II	SEQ ID No: 28
pBANspec	*NahRAsn169* *	SEQ ID No:97	Salicylic acid biosensor protein	SEQ ID No:100
	specR		Spectinomycin resistance protein	SEQ ID No: 98

* *nahRAsn169* gene [SEQ ID No: 99] encoding salicylic biosensor protein [SEQ ID No: 100] is located on the complementary strand to SEQ ID No: 97 at nucleotide positions: 102 - 1004.

A selected addicted strain (e3.18#1) and the non-addicted control *E. coli* strain (e3.18CON) were then tested for salicylic acid dependent growth by cultivation in a microtiter plate reader in liquid 2xYT medium supplemented with spectinomycin and kanamycin and presence/absence of salicylic acid (0 or 5 mM). As seen in figure 13, the control strain showed a relative reduction in growth rate and final biomass yield as a result of supplementation with salicylic acid, indicating that salicylic acid is toxic for growth. In contrast to the control strain, the growth rate of the addicted strain e3.18#1 was not reduced by the presence of salicylic acid, but rather the growth rate was higher in presence of 5 mM salicylic acid than the otherwise expected rate for non-addicted strains (Figure 14). The dependence of the addicted strain on a supply of salicylic acid for maximal growth (figure 14), confirms that the salicylic acid biosensor is able to induce the p_{SAL} promoter and the expression of dihydropteroate synthase, in the pathway leading tetrahydrofolate (vitamin B9) synthesis, which is essential for normal cell growth.

2.5 Benzoic acid addiction by regulated expression of an essential gene is shown to control bacterial cell growth

The native single genomic copy of the essential gene operon *folP-glmM* in the *E. coli* XL1 host strain was modified to allow its transcriptional control by the benzoic acid-responsive promoter p_{SAL} [SEQ ID No:118]. First, a gene encoding a benzoic acid biosensor (*Pseudomonas putida nahR)* (Table 12) was introduced into the XL1 host strain on the plasmid pBABspec. Then, replacement of the native *folP-glmM* promoter by the p_{SAL} promoter was engineered by means of lambda red-mediated recombineering.

Table 12 Benzoic acid addicted e3.22#4 clone

Plasmid/chromosome	**Gene**	**SEQ ID No:**	**Protein**	**SEQ ID No**
chromosome	*(FRT-kanR-FRT)-p_{SAL}-folP**	SEQ ID No:101	Dihydropteroate synthase	SEQ ID No:102
	*kanR***	SEQ ID No:27	Neomycin phosphotransferase II	SEQ ID No: 28
pBABspec	*NahR****	SEQ ID No: 103	Benzoic acid sensor protein	SEQ ID No: 106
	specR		Spectinomycin resistance protein	SEQ ID No: 104

*The native promoter of the chromosomal *folP – glmM* operon is replaced by the p_{SAL} promoter via the *(FRT-KanR-FRT)-p_{SAL}-folp* cassette [SEQ ID No.101]; which comprises a p_{SAL} promoter at position 1794 – 1929; a RBS at position 1930-1935, having the nucleotide sequence ACTTGC; and a portion of the *folp* gene. The nucleotide sequence of the complete *folp* gene in the *e3.22#4* strain is SEQ ID No: 87.

***kanR* gene [SEQ ID No: 27] encoding Neomycin phosphotransferase II [SEQ ID No: 28] is located on the complementary strand to SEQ ID No: 91 at nucleotide positions 389 - 1183.

****nahR* gene [SEQ ID No: 105] encoding benzoic biosensor protein [SEQ ID No:106] is located on the complementary strand to SEQ ID No: 103 at nucleotide positions: 102 - 1004.

Recombineering was performed as described in section 2.3 using well-described methods utilizing the pKD46 plasmid (Datsenko and Wanner, 2000)). Instead of gene disruption, a specific set of knock-out DNA fragments was used to only replace the native essential *folP-glmM* operon promoter and *folP* RBS. This set of knockout DNA fragments contained a kanamycin resistance cassette and the p_{SAL} promoter, as illustrated in the cartoon in figure 8, as well as a redundant nucleotide ("N", wherein "N" is any of A, T , G or C) in the RBS for *folP-glmM* to generate a four-membered library of *folP* translational strengths. This library allows selection of a translational strength for *folP* that suits the transcriptional regulation by the biosensor.

A non-addicted control *E. coli* strain (e3.22CON), also equipped with spectinomycin and kanamycin resistance genes, but wild-type control of *folP-glmM* expression (Table 13), was constructed in order to test the toxicity of benzoic acid.

Table 13 Benzoic acid non-addicted control e3.22CON

Plasmid/chromosome	**Gene**	**SEQ ID No:**	**Protein**	**SEQ ID No**
Chromosome	*kanR*	SEQ ID No:27	Neomycin phosphotransferase II	SEQ ID No: 28
pBABspec	*NahR*	SEQ ID No: 103	Benzoic acid sensor protein	SEQ ID No: 106
	specR		Spectinomycin resistance protein	SEQ ID No:104

**kanR* gene [SEQ ID No: 27] encoding Neomycin phosphotransferase II [SEQ ID No: 28].

***nahR* gene [SEQ ID No: 105] encoding benzoic biosensor protein [SEQ ID No:106] is located on the complementary strand to SEQ ID No: 103 at nucleotide positions: 102 - 1004.

Finally, candidate recombineered cells were selected for by plating on LB agar plates supplemented with 5 mM benzoic acid and spectinomycin for maintenance of pBABspec and kanamycin for selection of inserted knockout fragment.

A selected addicted strain (e3.22#4) and the non-addicted control *E. coli* strain (e3.18CON) were then tested for benzoic acid dependent growth by cultivation in a microtiter plate reader in liquid 2xYT medium supplemented with spectinomycin and kanamycin and w/wo salicylic acid (0 or 5 mM). As seen in Figure 15, the control strain showed a relative reduction in growth rate and final biomass yield as a result of supplementation with benzoic acid, indicating that benzoic acid is toxic for growth. In contrast to the control strain, the growth rate and final biomass yield of the addicted strain e3.18#1 was enhanced by supplementation with benzoic acid with a growth rate higher than otherwise expected for non-addicted strains (Figure 16).

The dependence of the addicted strain on a supply of benzoic acid for maximal growth (figure 16), confirms that the benzoic acid biosensor is able to induce the p_{SAL} promoter and the expression of dihydropteroate synthase, in the pathway leading tetrahydrofolate (vitamin B9) synthesis, which is essential for normal cell growth.

2.6 General scheme for engineering and optimization of addiction to effector metabolite by regulated expression of an essential gene

As exemplified in the previous examples, biosensors can be engineered into cells to control growth by addiction to an effector metabolite produced by the cell. The degree of addiction, which results in a relative growth advantage for cells that produce and accumulate the effector metabolite, may be increased by reducing the growth of an addicted cell in which the cellular levels of effector metabolite are below a given threshold. The degree of addiction of the addicted cell can be controlled by regulating the basal expression of the biosensor and/or the regulated essential gene.

2.6.1 The degree of growth reduction for a given host cell that fails to produce sufficient effector metabolite can be increased by providing the regulated essential gene with a weaker RBS sequence e.g. generated with the "RBS Calculator" (https://salislab.net/software/), thereby reducing its rate of translation. Alternatively, the basal transcription strength of the regulated essential gene can be reduced by providing a biosensor-regulated, but weaker, promoter to drive its expression. Inversely, the degree of addiction can be reduced, to minimize the growth reduction for non-producing cells, by employing a stronger RBS sequence or stronger promoter to enhance basal expression of the regulated essential gene.

2.6.2 The biosensor gene, engineered into the cell, is expressed under the control of a constitutive promoter. A suitable promoter can be selected from the set of the exemplary constitutive promoters provided in SEQ ID: 47-65. A suitable promoter is one that drives expression of the biosensor in an amount that is responsive to the effector metabolite concentration, while avoiding constitutive saturation of the promoter of the essential gene. Preferably, the engineered addicted cell of the invention is one whose growth rate is not reduced (no fitness cost) so long as the cell produces the effector metabolite at or above a predetermined amount.

2.7 Method for engineering host cell addiction to its metabolic product using tailor-made biosensor:promoter pairs

A large number of biosensors are described in the literature, in particular in the RegPrecise and Genbank databases, each being characterized for its specific detection of one of a wide range of metabolic products of interest, and which are suitable for engineering host cell addiction to a particular effector metabolite of interest.

By way of example, a list of suitable biosensor and cognate promoter pairs is given in Table 13 for respective effector metabolites, that are suitable for engineering host cell addiction, and for regulating the expression of a gene in the host cell required for its growth and/or survival. A host cell addicted to one of the effector metabolites listed in Table 13 can be constructed using the corresponding biosensor:promoter pairs to regulate the expression of the essential gene *folP-glmM,* as follows. The native *folP-glmM* promoter in the selected host strain is replaced by the respective promoter listed in Table 13, by means of lambda red-mediated recombineering as described in section 2.3 for insertion of the p_{BAD} promoter. Further, the gene encoding the respective biosensor listed in Table 13, is introduced into the selected host strain on a plasmid, as described for the introduction of the plasmid pBALspec comprising the triacetic acid lactone biosensor in Example 2.3. Standard molecular expression tuning may additionally be employed to optimize the degree of addiction as described above.

Table 13 Specific implementations of biosensors for diverse effectors

Effector metabolite	Promoter	Promoter name	Biosensor name	Biosensor nucleotide sequence	Biosensor protein sequence	Effector metabolite conc. (µM)
Phenol	SEQ ID No:107	dmpRKLM	DmpR	SEQ No:108	SEQ No:109	1000
2-Amino-biphenol	SEQ No:110	hbpC	HbpR	SEQ No:111	SEQ No:112	100
Catechol	SEQ No:107	dmpRKLM	CapR	SEQ No:113	SEQ No:114	100
D-glycerate	SEQ No:115	gudP	CdaR	SEQ No:116	SEQ No:117	750
triacetic acid lactone	SEQ ID No: 66	p_{BAD}	AraCtal	SEQ ID No:95	SEQ ID No:96	
salicylic acid	SEQ ID No: 118	p_{SAL}	NahR_Asn169	SEQ ID No:99	SEQ ID No:100	
benzoic acid	SEQ ID No: 118	p_{SAL}	NahR	SEQ ID No:105	SEQ ID No:106	

Example 3. Riboswitch biosensors extend the range of addiction molecules for regulating cell growth.

3.1 Use of thiamine pyrophosphate (TPP)-sensitive riboswitch-controlled TA system to regulate bacterial cell growth

A TPP-responsive riboswitch (ThiMN15#19) was engineered into the previously constructed L-arabinose-responsive TA-based correction system (pBAD-TA5 to yield pBAT-TA5). This TPP riboswitch functions as an "ON-type" when in the presence of its ligand TPP, and it is functional at the level of translation. The riboswitch was inserted into the DNA region of the pBAD-TA5 gene construct that encodes the 5' untranslated region (UTR) of the antitoxin mRNA sequence (figure 17). For optimal functionality of the riboswitch, a protein-encoding sequence *tet* was also added as N-terminal fusion to YefM as it originated from the screen where ThiMN15#19 had been identified. An alternative TPP-responsive riboswitch was constructed, where an "OFF-type" riboswitch was inserted into the DNA region of the pBAD-TA5 gene construct that encodes the 5'-UTR of the toxin-encoding sequence to yield the same ligand-responsive growth.

Table 14 listing the genetic features of the TPP-addicted-TA-regulated *E. coli* strain based on the *E. coli* XL1 parent strain

Table 14

Plasmid/ chromosome	**Gene**	**SEQ ID No:**	**Protein**	**SEQ ID No**
pBAT-TA5	*araC**	SEQ ID No: 31	AraC	
	*Thi-tet-yefM***		Tet-YefM	SEQ ID No: 32
	yoeB		YoeB	SEQ ID No: 33
	ampR		Beta-lactamase	SEQ ID No: 34

**araC* gene [SEQ ID No: 5] encoding araC biosensor protein [SEQ ID No: 6] is located on the complementary strand to SEQ ID No: 31 at nucleotide positions: 96 – 974;

***thi* riboswitch is located at nucleotides 1291 - 1452 of SEQ ID No.: 31

A modified *E. coli* strain comprising the TPP responsive-riboswitch-regulated TA correction system (pBAT-TA5) showed enhanced growth in the presence of

the inducer TPP, thereby demonstrating functionality of the TPP-addiction mediated via a riboswitch and TA system (figure 18). An alternative TPP responsive-riboswitch-regulated TA correction system was engineered with a constitutive promoter driving expression of the riboswitch-*yefM* mRNA. From a selection of constitutive promoters (SEQ ID Nos.: 47-65, 89) of different strengths, the p_{BAD} promoter of pBAT-TA5 was replaced and the resulting plasmids transformed into *E. coli* XL1. These were tested to identify plasmids where the strength of the constitutive promoter allowed the riboswitch-yefM transcript to cause good TPP-conditional growth of the transformed *E. coli* cell, i.e. when grown in liquid medium with and without addition of 500 µM TPP.

3.1 Use of a TPP-sensitive riboswitch-regulated TA system to enhance small molecule production in bacteria.

In order to demonstrate that modified *E. coli* strain comprising the TPP riboswitch (ThiMN15#19) TA system, was dependent on internal TPP overproduction, the cells were further genetically modified to biosynthesize higher concentrations of TPP, which were compared with a reference strain cells retaining the wild-type intracellular TPP accumulation level. Biosynthesis of elevated TPP concentration was engineered by translational deregulation of the chromosomal native *E. coli thiC* gene. This was achieved by introducing a point mutation (by substituting wild-type nucleotide residue A at position (-135), with nucleotide B (= any one of C, G and T) in the region of the native *E. coli thiC* gene [GeneID:948492], to give the following 142nt sequence located directly upstream of the *thiC* open reading frame, corresponding to the transcribed 5'-UTR:
ATTCGGG**B**TCCGCGGAACCTGATCAGGCTAATACCTGCGAAGGGAACAAGAGTTAATCTGCTA TCGCATCGCCCCTGCGGCGATCGTCTCTTGCTTCATCCGTCGTCTGACAAGCCACGTCCTTAA CTTTTTGGAATGAGCT [SEQ ID No: 90] which when transcribed yields a mutant *E. coli* riboswitch transcript, that is insensitive to TPP feedback regulation. Growth and TPP production by these *E. coli* strains comprising, or lacking, the TPP-sensitive riboswitch-regulated TA gene correction system in the modified and reference stains, are then compared.

Example 4 Improved fermentation productivity through use of product-addicted strain based on control of essential genes

A plasmid (pMEV7C), encoding the genes for the metabolic pathway to mevalonate, was inserted in *E. coli* XL1. The strain was further engineered to encompass a biosensor-based addiction module according to the invention e.g. by following the methods for construction of strain *e3.9* comprising the plasmid (*FRT-kana-FRT-*p_{BAD}*-RBS1)-folP-glmM*), where growth requires expression of an essential gene (see Example 2.2). As a control, the same pathway plasmid (pMEV7C) was inserted in an *E. coli* XL1 strain to generate *pe1* only differing in way of genetic engineering from *e3.9* (Table 3) by the fact that no genetic changes had been introduced on the chromosome (i.e. the expression of essential genes were not linked to presence of the metabolic pathway product).

The two strains *e3.9* and *pe1* were grown at 37 deg. C with 250-300 rpm horizontal shaking in 2xYT medium (with 30 µg/mL chloramphenicol and 50 µg/mL spectinomycin) for 55 cell generations to simulate a fermentation of large industrial size. This generation number was obtained in 25 mL shake flask cultures by transferring ≥ 0.5 vol% culture to fresh 2xYT medium an appropriate number of times according to the cell densities measured when transferring the culture. Final productivity of the cell population was evaluated by taking a sample for HPLC analysis following culturing at 37 deg. C. for 72 hours after the final transferring. As seen in figure 19, the concentration of mevalonate (MVA) in the control strain *pe1* was only 35 % of the concentration in the *e3.9* strain, demonstrating the advantage of linking product-presence to growth of the strain during cultivations with many cell divisions.

A high generation number could be reached using a continuous chemostat fermentor, in which the cells are constantly dividing through inlet of new medium and outlet of culture. Optionally, the strains could also be cultured at another temperature such as 30 deg. C. The productivity of the cells could further be measured at various numbers of generations such as (but not limited to) 20, 30, 40, 50, 60, 70 and 150 generations. To evaluate the stability of the pathway over time (cell generations), the cells can be re-

cultured from a stored sample of the generation of cells in a 25% glycerol stock at -80 degrees Celsius. This sample could be re-cultured in 15 mL 2xYT (with 30 µg/mL chloramphenicol and 50 µg/mL spectinomycin) under otherwise same culture conditions and measuring the productivity (according to method described in previous section). To evaluate whether genetic mutations were less predominant in the product pathway of the product-addicted strain, DNA sequencing of the strains could be performed.

The cultivations can be carried out in growth medium, which can direct higher mevalonate productivity, such as M9 minimal medium supplemented with 0.4-4 (w/v)% glucose.

Mevalonate production (in the culture medium) was detected by HPLC, following treatment of 300 µL sampled culture broth with 23 µL 20 % sulfuric acid for conversion to the mevalonolactone form. The mixture was vortexed and cells separated from the medium by centrifugation at 17,000 g for 3 mins. 30 µL supernatant (medium) was injected in an Ultimate 3000 HPLC running with a 5 mM sulfuric acid mobile phase (0.6 mL/min) on an Aminex HPX-87H ion exclusion column (300 mm×7.8 mm, Bio-Rad Laboratories, Hercules, CA, USA) at 50 °C, with detection using refractive-index (RI) channel.

Example 5 Improved fermentation productivity through use of product-addicted strain based on control of toxin-antitoxin systems

A plasmid (pMEV7C) encoding the genes for the metabolic pathway to mevalonate was inserted in *E. coli* XL1. The strain was further engineered to encompass a biosensor-based addiction module according to the invention e.g. by following the methods for construction of strain *m29*, which comprises (pBAM-TA5), where growth is controlled by expression of the anti-toxin and toxin pair (see Example 1.4). As a control, the exact same pathway plasmid was inserted in an *E. coli* XL1 strain, to create a strain only differing from *m29* by the fact that a plasmid featuring the invented system had not been introduced (i.e. the expression of antitoxin was not linked to presence of the metabolic pathway product).

Pre-cultures in 2xYT were inoculated from single colonies of the two strains, and main cultures were inoculated from these when OD600 (measured on 200 µL sample in a BioTek SynergyH1 plate reader) was 0.1-0.5. From there, the two strains were grown at 30 deg. C with 250-300 rpm horizontal shaking in 2xYT medium for 70 cell generations to simulate a fermentation of large industrial size. This generation number was obtained in 50 mL shake flask cultures by transferring ≥ 0.5 vol% culture to fresh medium an appropriate number of times according to the cell densities measured before transferring the culture. Optionally, the generation number could be reached using a continuous chemostat fermentor, in which the cells are constantly dividing through inlet of new medium and outlet of culture. The productivity of the cells was measured at various numbers of generations such as (but not limited to) 20, 30, 40, 50, 60 and 70 generations. This was done by recording a sample of the generation of cells in a 25% glycerol stock stored at -80 deg. C and re-culturing them in tubes with 15 mL 2xYT under otherwise same culture conditions and measuring the productivity (according to method described in Example 4).

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Claims

1. A genetically modified microbial cell for intracellular biosynthesis of a cellular metabolite comprising:
   a. a first nucleic acid molecule wherein the transcription and/or translation of said molecule yields a biosensor capable of binding said cellular metabolite to form a complex;
   and
   any one selected from the group consisting of:
   b. a second nucleic acid molecule comprising a coding sequence encoding a first protein required for cell growth and/or survival, wherein the second nucleic acid molecule is operably linked to a first promoter;
   wherein expression of said first protein encoded by said second nucleic acid molecule is induced when said biosensor and said cellular metabolite form a complex;
   c. a second nucleic acid molecule comprising a coding sequence encoding a first protein required for cell growth and/or survival, wherein the second nucleic acid molecule is operably linked to a first promoter; and
   a third nucleic acid molecule encoding a second protein that is toxic for cell growth and/or survival, wherein said third nucleic acid molecule comprises a coding sequence operably linked to a second constitutive promoter;
   wherein expression of said first protein encoded by said second nucleic acid molecule is induced when said biosensor and said cellular metabolite form a complex; and
   d. a second nucleic acid molecule encoding a protein that is toxic for cell growth and/or survival, wherein said second nucleic acid molecule comprises a coding sequence operably linked to a promoter; wherein expression of said protein is prevented when said biosensor and said cellular metabolite form a complex;

   whereby arrest of growth and /or death of said cell due to an absence of complex formation does not depend on externally

supplied growth inhibitor or growth retardant.

2. The genetically modified microbial cell of claim 1 according to (b), wherein
   - said first promoter is an inducible promoter, and
   - said encoded first protein is essential for growth of the cell.

3. The genetically modified microbial cell of claim 1 according to (c), wherein:
   - said second protein is a toxin; and
   - said first protein is an anti-toxin protein cognate to said toxin protein; and
   - said first promoter is inducible, and

   wherein said biosensor is a transcription factor capable of binding to said metabolite to form a complex, and wherein said complex is capable of binding to said first inducible promoter.

4. The genetically modified microbial cell of claim 1 according to (c), wherein:
   - said second protein is a toxin; and
   - said first protein is an anti-toxin protein cognate to said toxin protein; and
   - said first nucleic acid molecule is operably linked to said second nucleic acid molecule upstream to the coding sequence and is operably linked downstream of the first promoter, wherein the first promoter is a constitutive promoter, and

   wherein said biosensor obtained on transcription of said first nucleic acid molecule is a riboswitch capable of binding to said metabolite to form a complex.

5. The genetically modified microbial cell of claim 1 according to (d), wherein:
   - said protein is a toxin; and
   - said promoter is inducible; and

wherein said biosensor is a transcription factor capable of binding to said metabolite to form a complex, and wherein said complex is capable of binding to said inducible promoter.

6. The genetically modified microbial cell of claim 1 according to (d), wherein:
   - said protein is a toxin;
   - said first nucleic acid molecule is operably linked to said second nucleic acid molecule upstream to the coding sequence and is operably linked downstream of the first promoter, and wherein the first promoter is a constitutive promoter, and

     wherein said biosensor obtained on transcription of said first nucleic acid molecule is a riboswitch capable of binding to said metabolite to form a complex.

7. The genetically modified microbial cell of claim 3 or 4,
wherein the amino acid sequence of said antitoxin has at least 80% sequence identity to a sequence selected from the group consisting of SEQ ID No.: 2, 38, 42, and 46; and
wherein the amino acid sequence of said cognate toxin protein has at least 80% sequence identity to a sequence selected from the group consisting of SEQ ID No.: 3, 36, 40 and 44 respectively.

8. The genetically modified microbial cell of claim 5 or 6,
wherein the amino acid sequence of said toxin protein has at least 80% sequence identity to a sequence selected from the group consisting of SEQ ID No.: 3, 36, 40 and 44 respectively.

9. The genetically modified microbial cell of any one of claims 1 - 3, 5, 7 and 8,
wherein said biosensor obtained on transcription of said first nucleic acid molecule is a riboswitch capable of binding to said metabolite to form a complex, and
wherein the nucleic acid sequence of said a first nucleic acid molecule

is selected from the group consisting of: nucleotides sequence 1291 - 1452 of SEQ ID No.: 31; SEQ ID No.:69, 70 and 71.

10. The genetically modified microbial cell of claim 1 – 3 and 5, wherein said biosensor obtained on translation of said first nucleic acid molecule is a transcription factor capable of binding to said metabolite to form a complex, and
wherein the nucleic acid sequence of said a first nucleic acid molecule is selected from the group consisting of: SEQ ID No.:6, 12 and 68.

11. The genetically modified microbial cell of claim 2,
wherein the amino acid sequence of said essential protein has at least 80% sequence identity to a sequence selected for the group consisting of: SEQ ID No.: 76, 78, 80, 82, 84, 86 and 88.

12. The genetically modified microbial cell of any one of claims 1 - 11, wherein the cellular metabolite is selected from the group consisting of: isoprenoid(s), vitamin(s), carboxylic acid(s), amino acid(s), fatty acid(s), alcohol(s), and polyketide(s).

13. A method of genetically modifying a microbial cell for the biosynthesis of a metabolite comprising the steps of introducing into the cell:
- a nucleic acid molecule encoding a toxin operably linked to a constitutive promoter; and
- a nucleic acid molecule encoding an anti-toxin cognate to the toxin, wherein the molecule is linked to a inducible promoter; and
- a nucleic acid molecule wherein the transcription and/or translation of said molecule yields a biosensor capable of binding to the metabolite;
wherein expression of said antitoxin is induced when said biosensor and said cellular metabolite form a complex; and
wherein arrest of growth and /or death of said cell due to an absence of complex formation does not depend on externally

supplied growth inhibitor or growth retardant.

14. A method for producing a biosynthetic metabolite comprising the steps of:

a) providing a genetically modified microbial cell according to any one of claims 1 - 12,

b) introducing the genetically modified microbial cell into a cultivation medium comprising a substrate for production of said metabolite, and

c) recovering metabolite produced by said culture,

wherein a lack of metabolite production in said genetically modified microbial cell or progeny cell thereof attenuates multiplication of said cell as compared to a non-genetically modified parent cell from which said modified microbial cell was derived.

15. Use of a genetically modified microbial cell according to any one of claims 1 - 12, for producing a biosynthetic metabolite, wherein a lack of metabolite production in said genetically modified microbial cell or progeny cell thereof attenuates multiplication of said cell as compared to the metabolite producing genetically modified microbial cell.

Figure 1

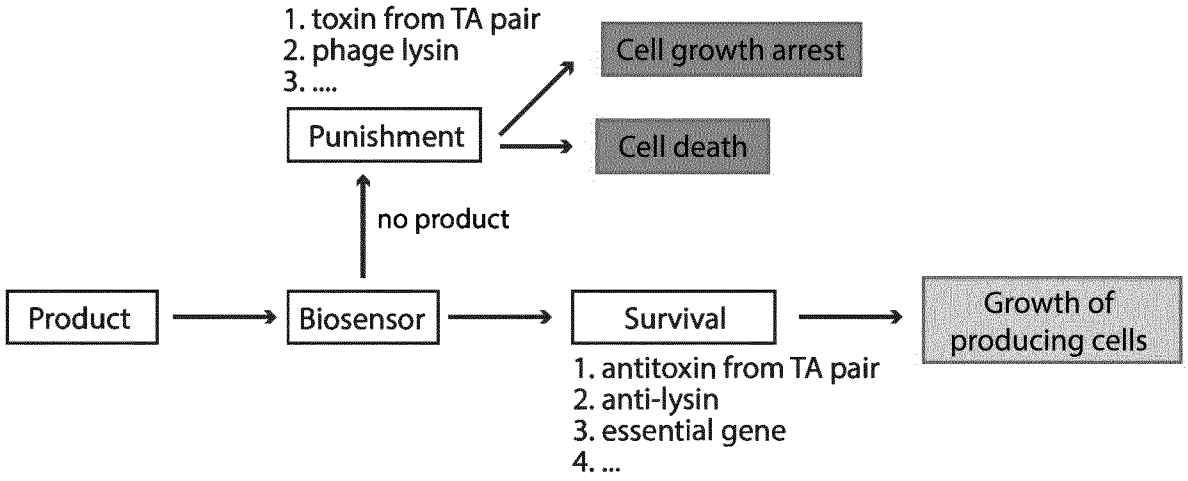

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Figure 2

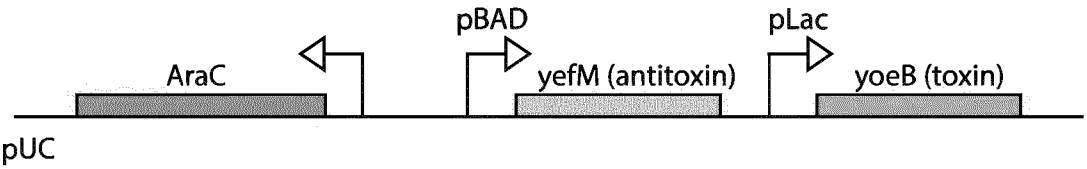

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Figure 3A

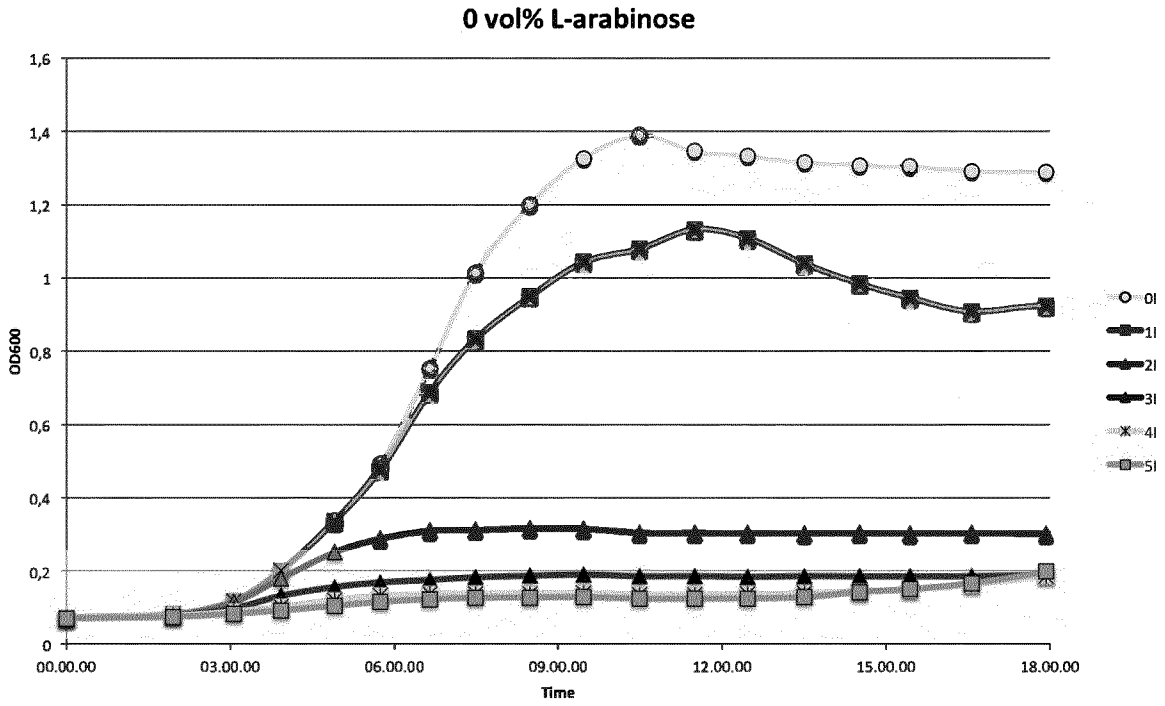

Figure 3B

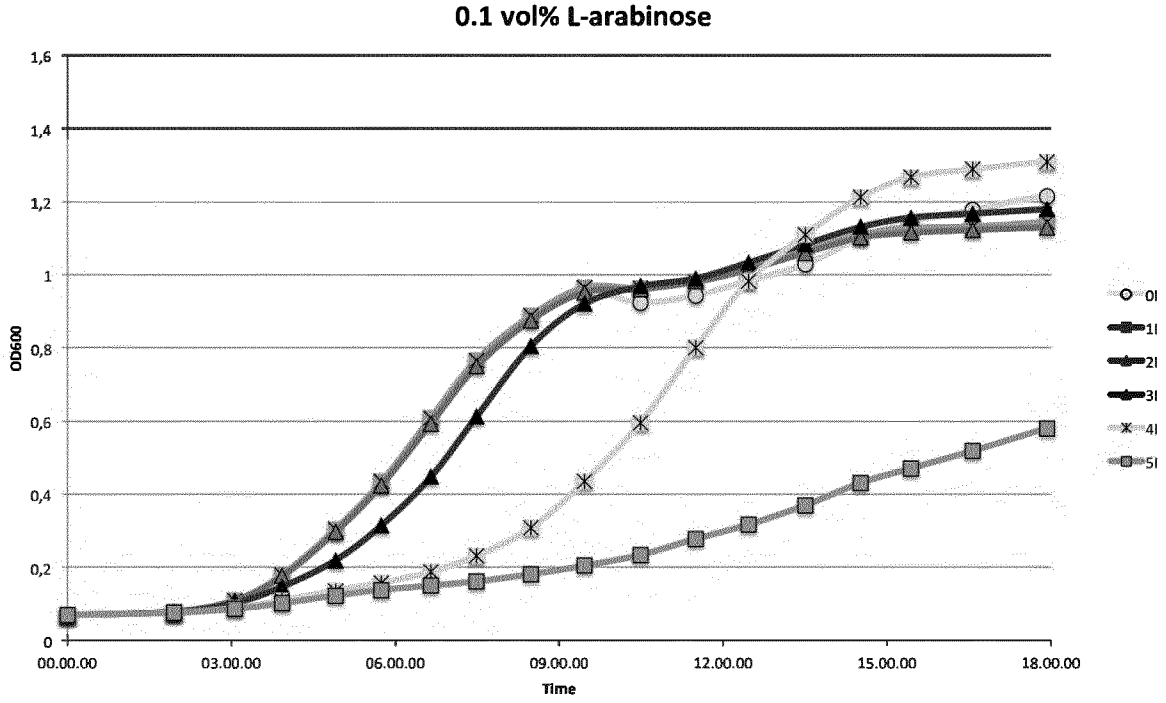

Figure 4

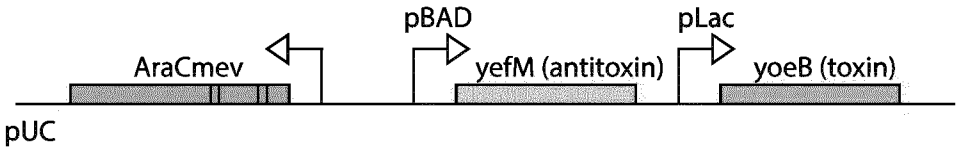

Figure 5

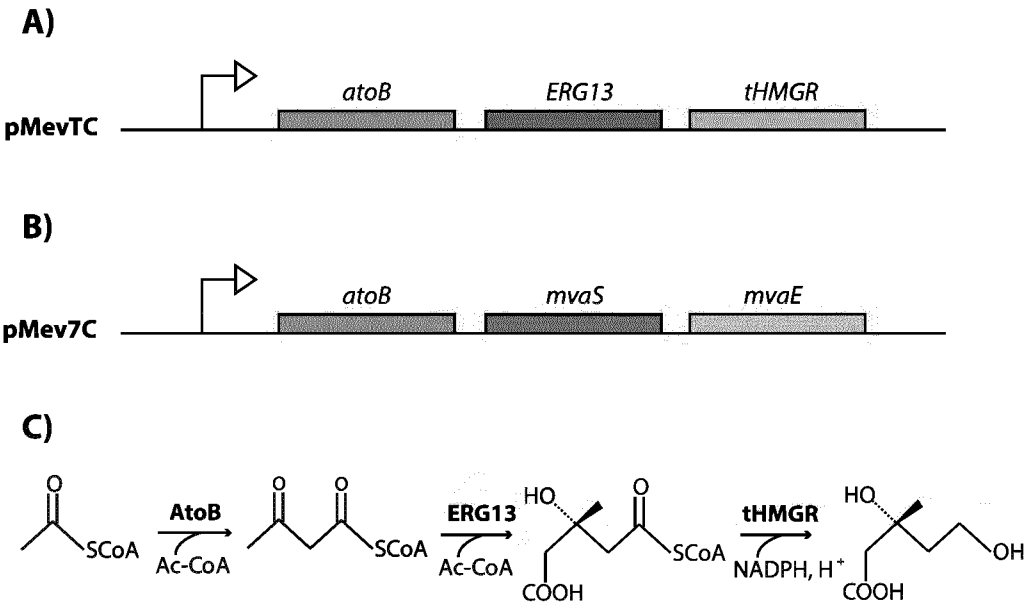

Figure 6A

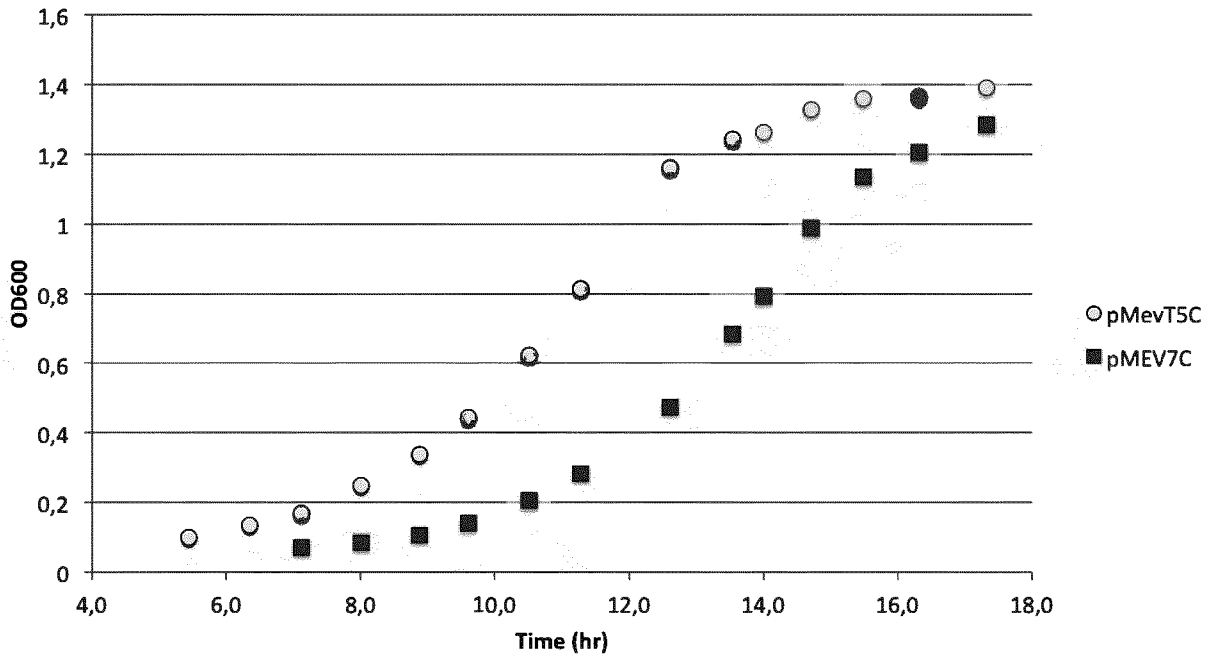

Figure 6B

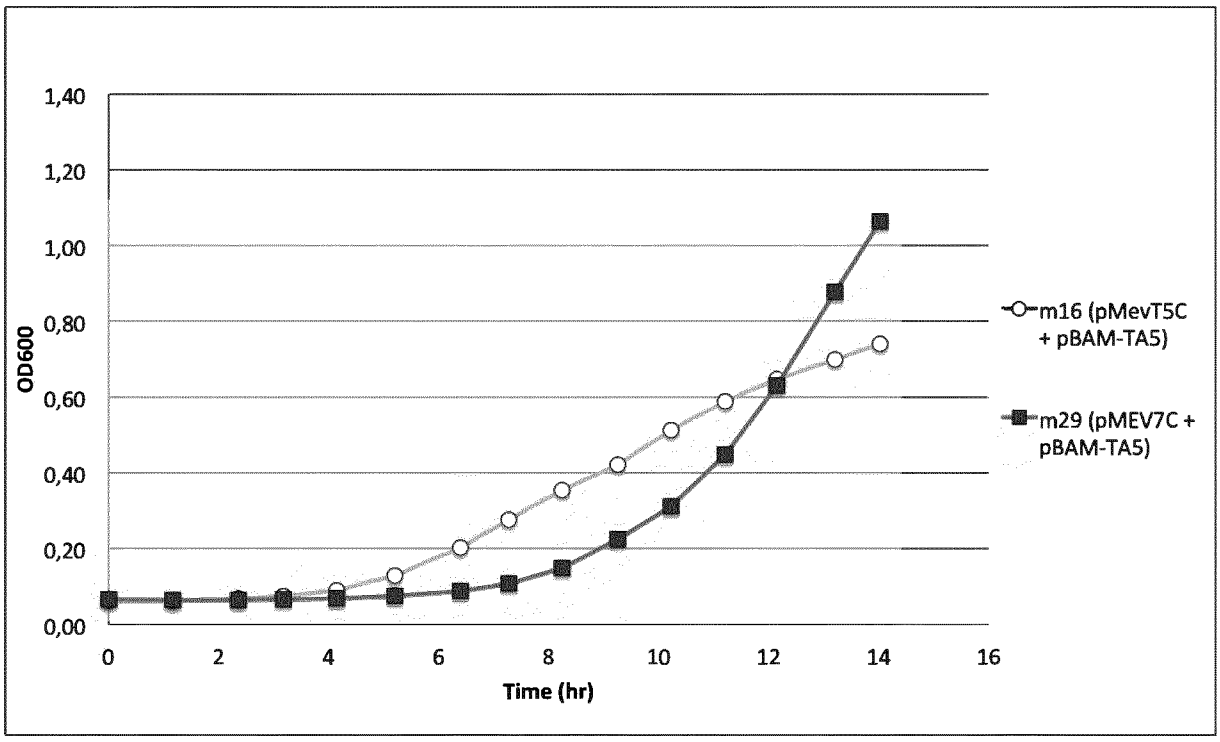

Figure 7

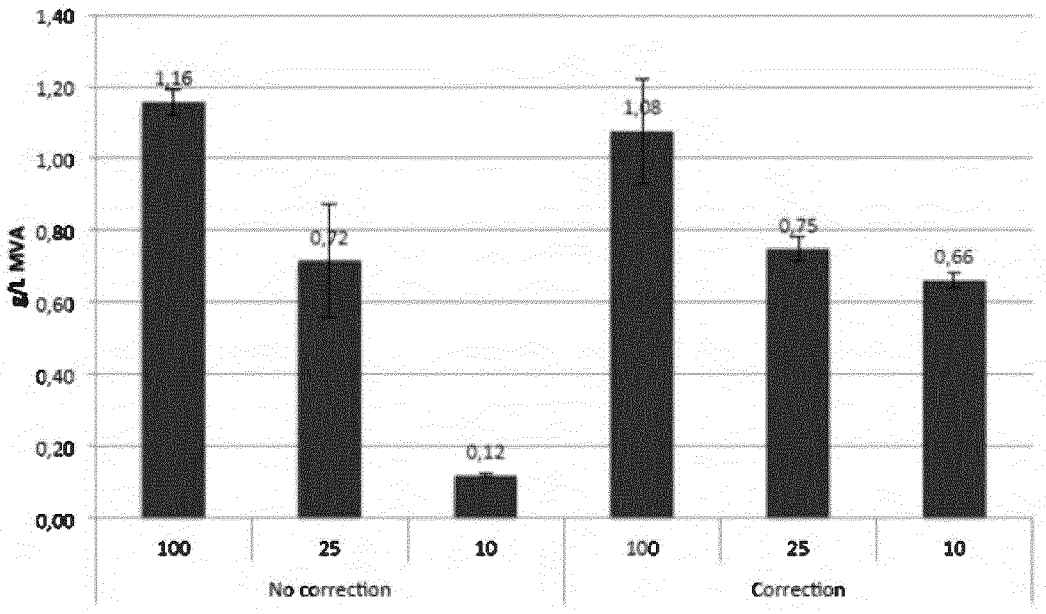

Figure 8

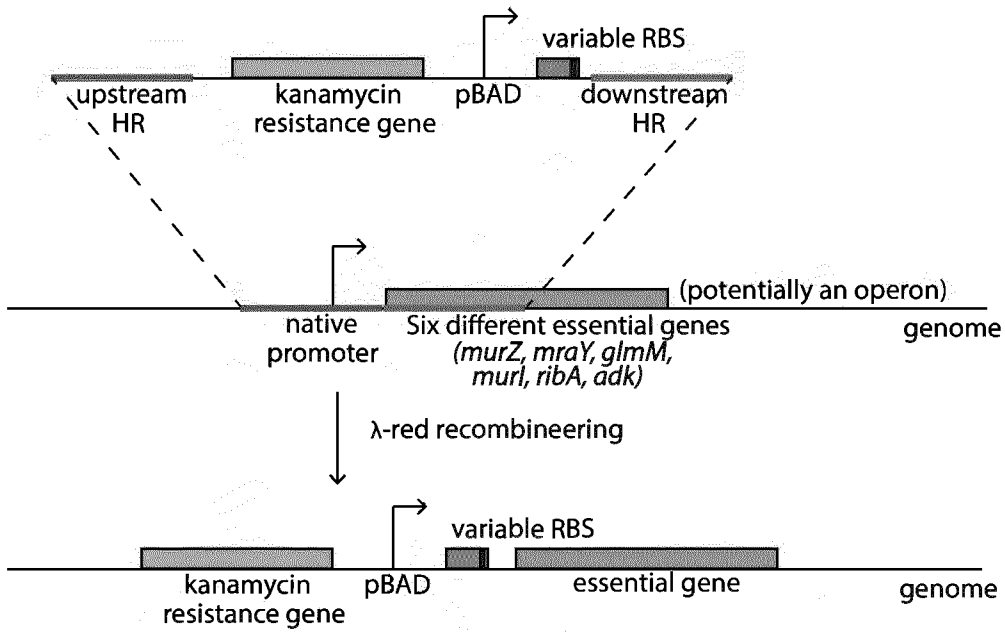

Figure 9

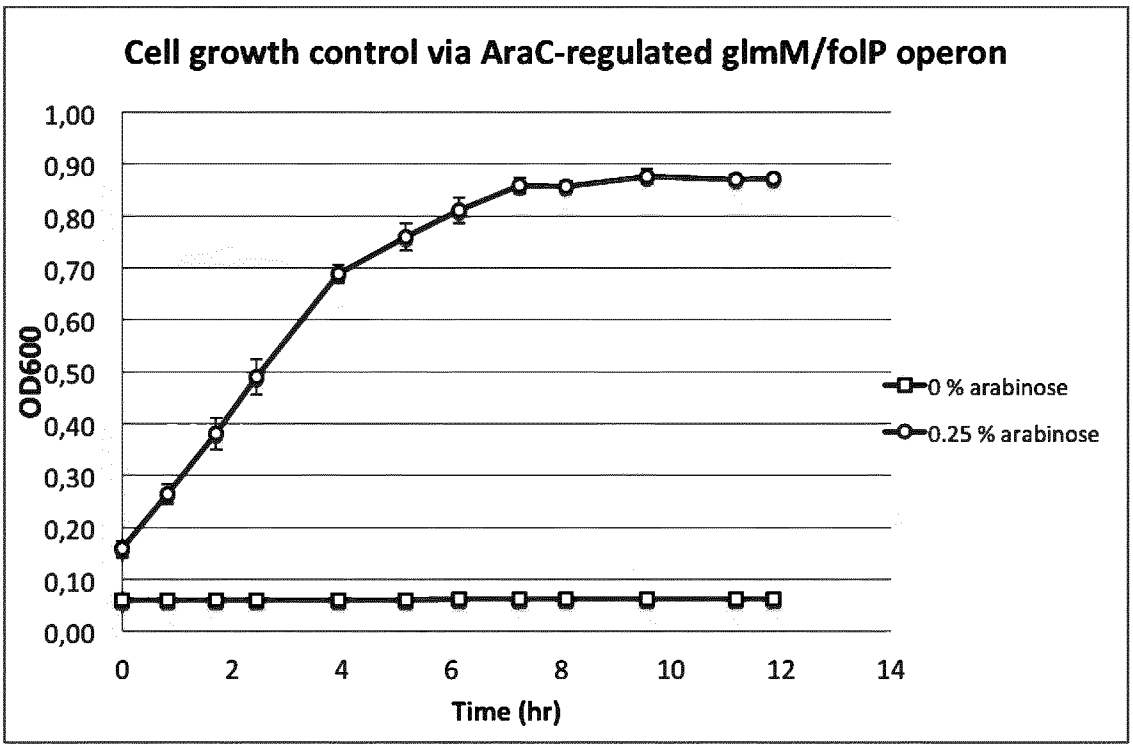

Figure 10

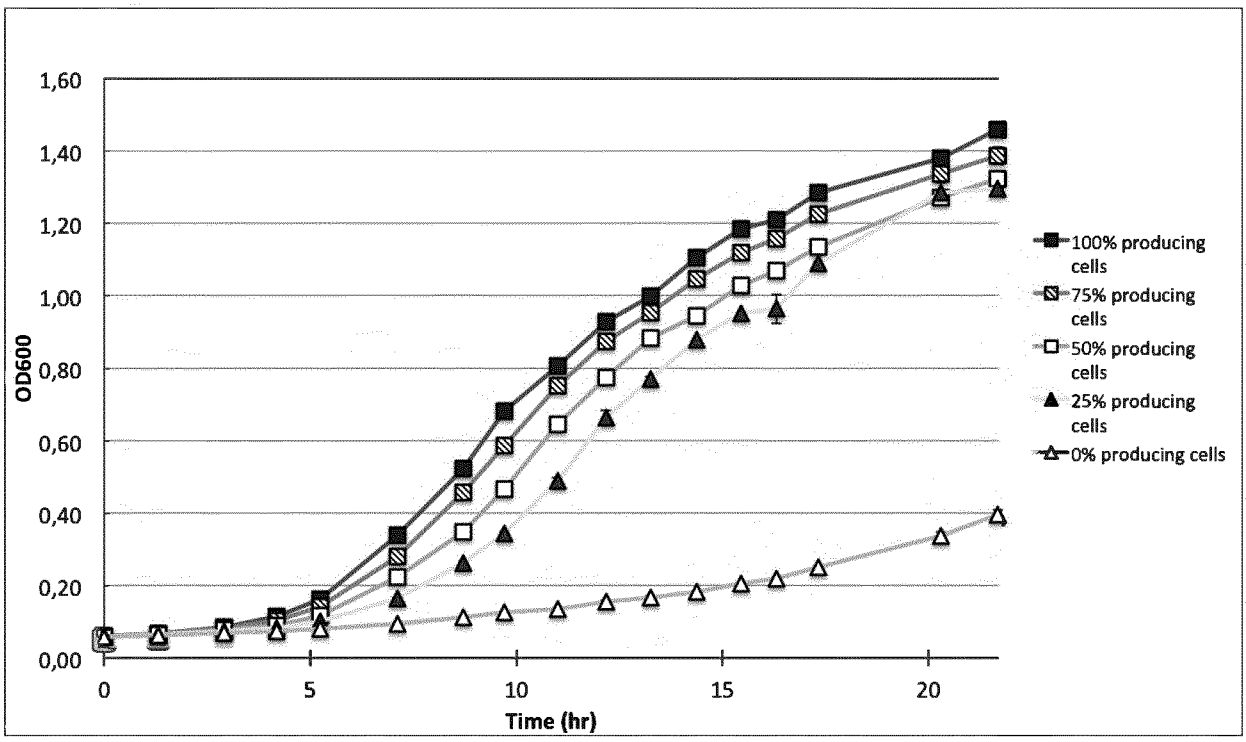

Figure 11

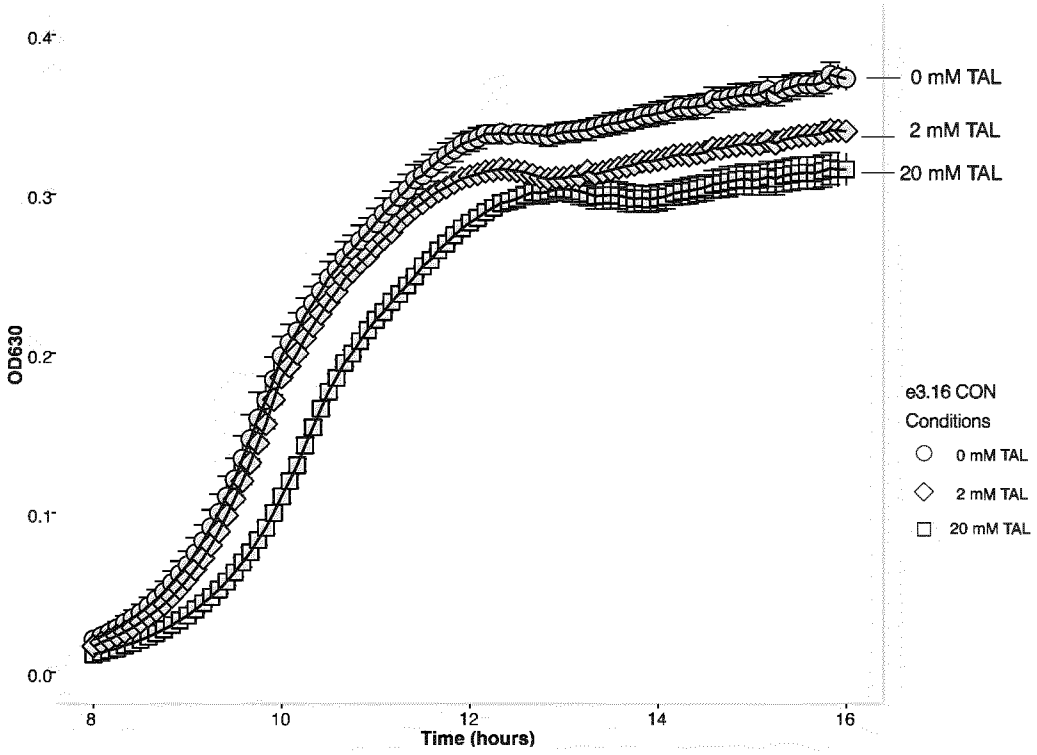

Figure 12

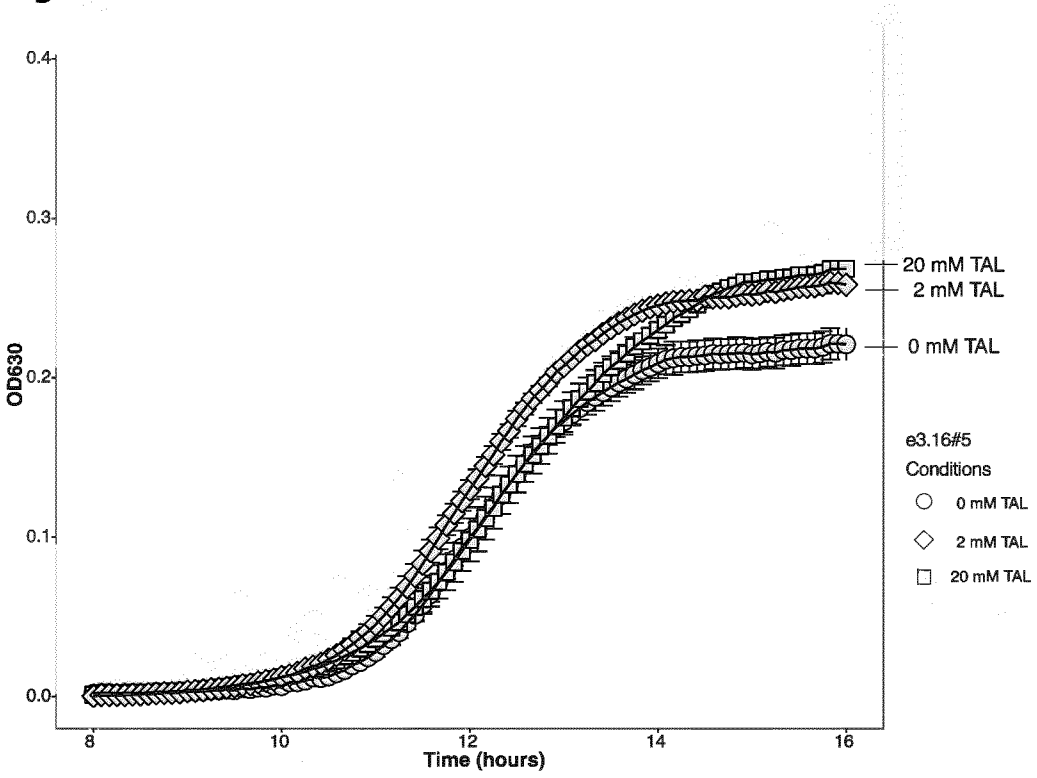

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Figure 13

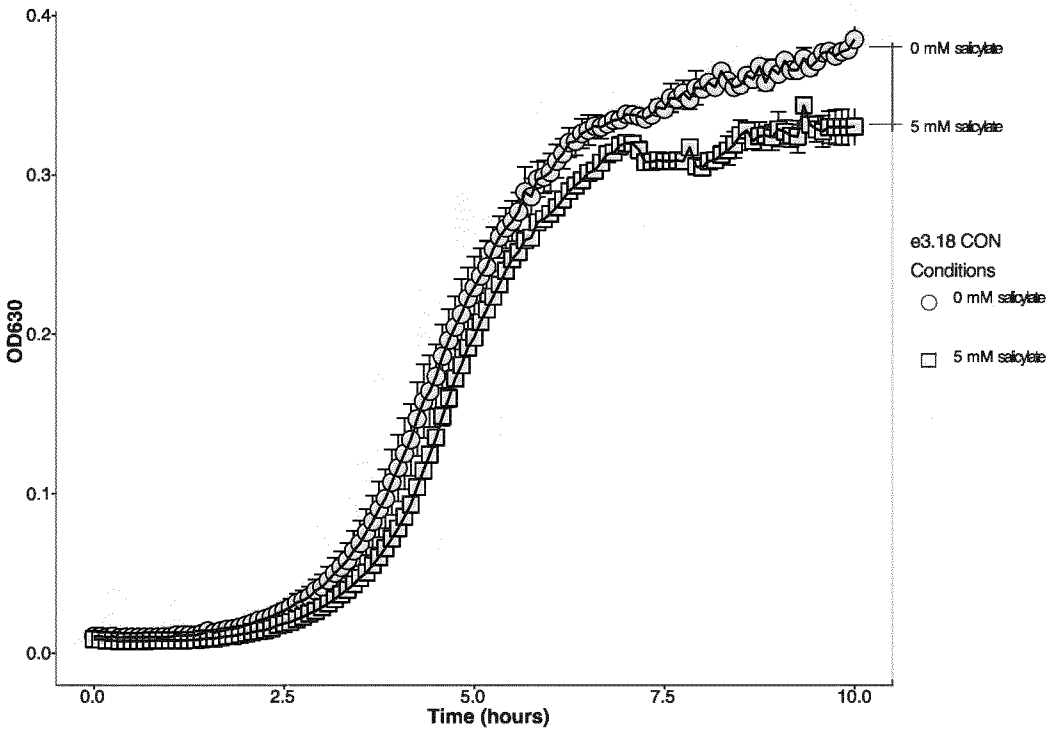

Figure 14

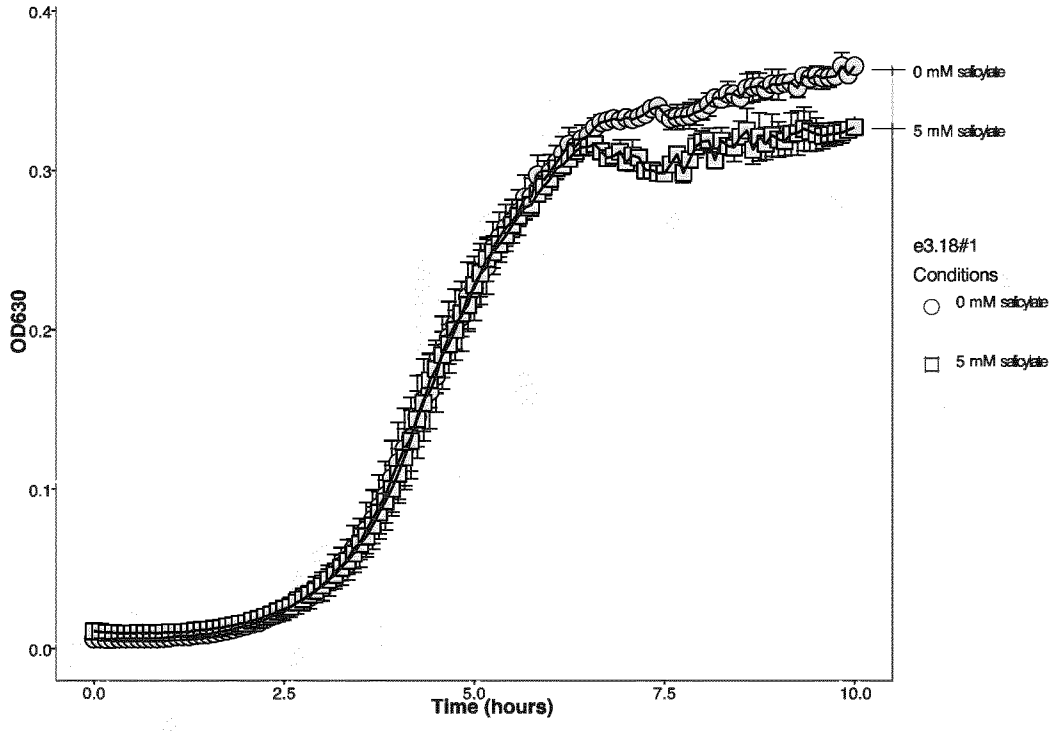

Figure 15

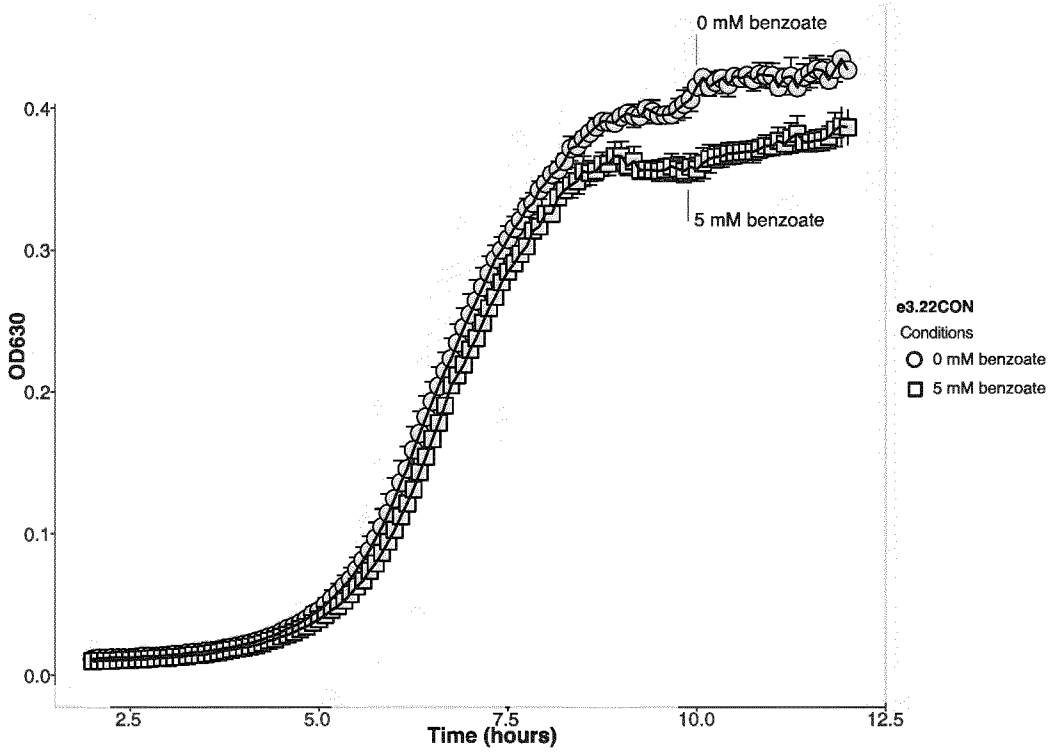

Figure 16

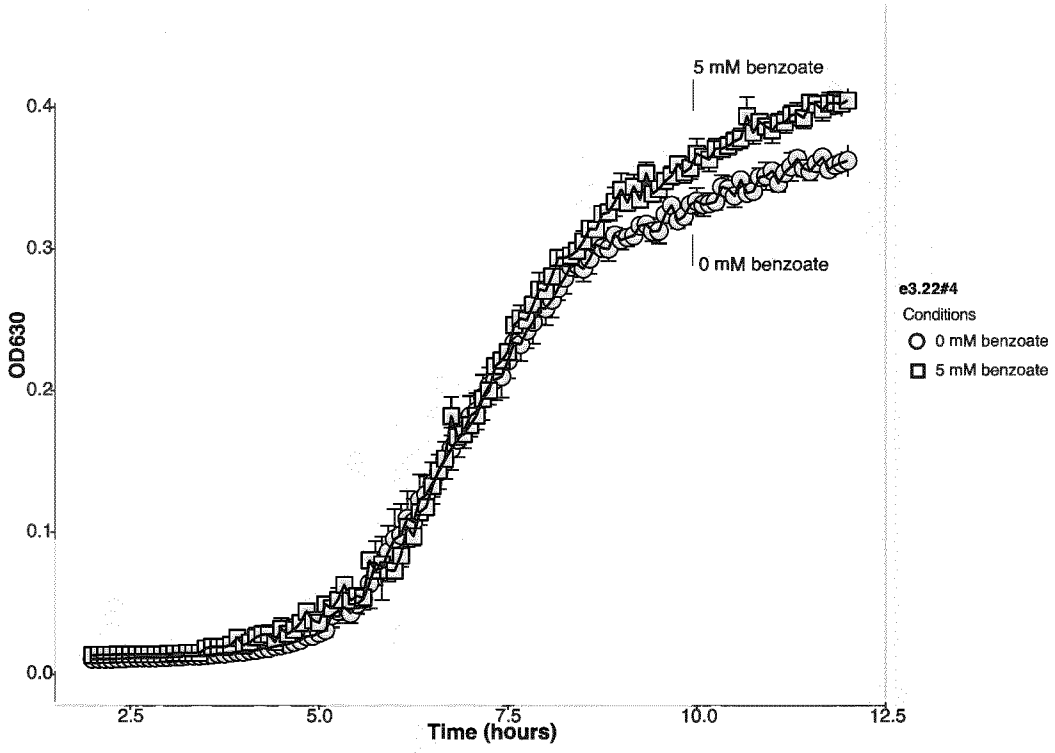

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Figure 17

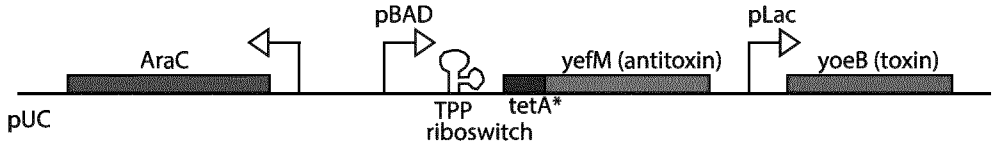


Figure 18

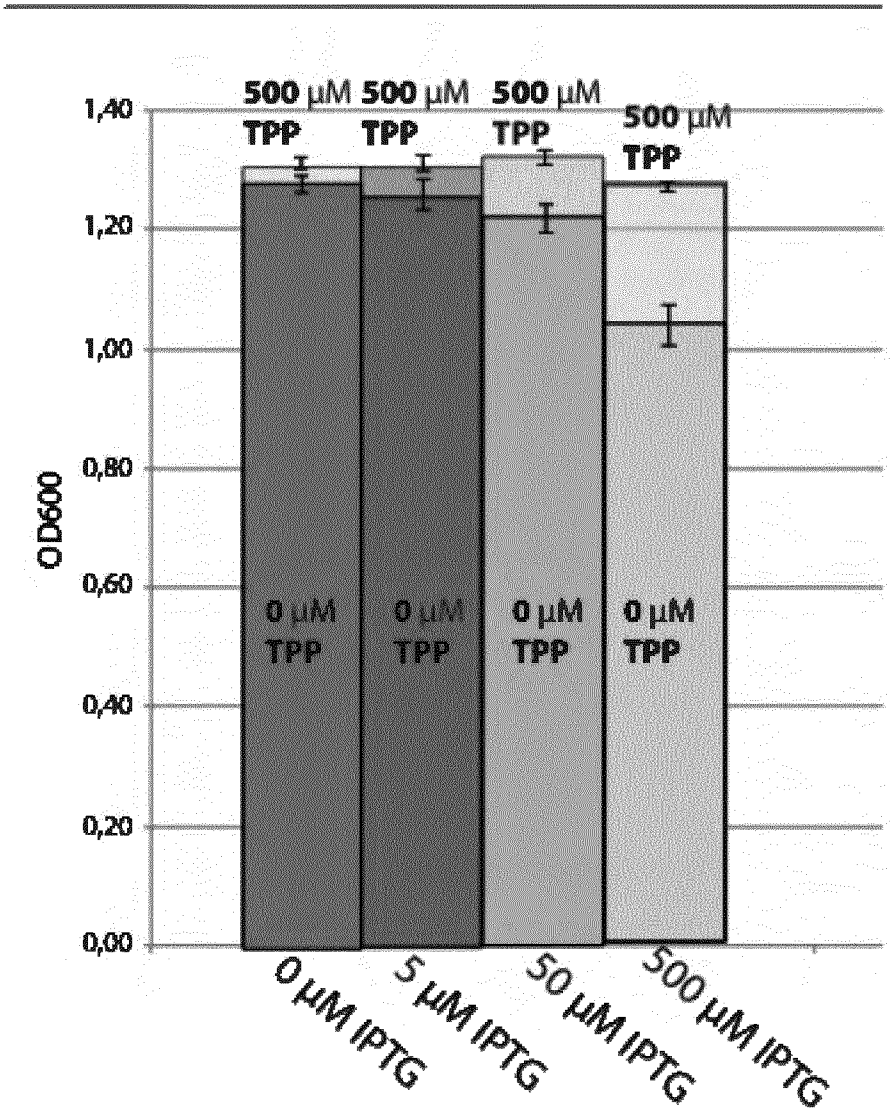
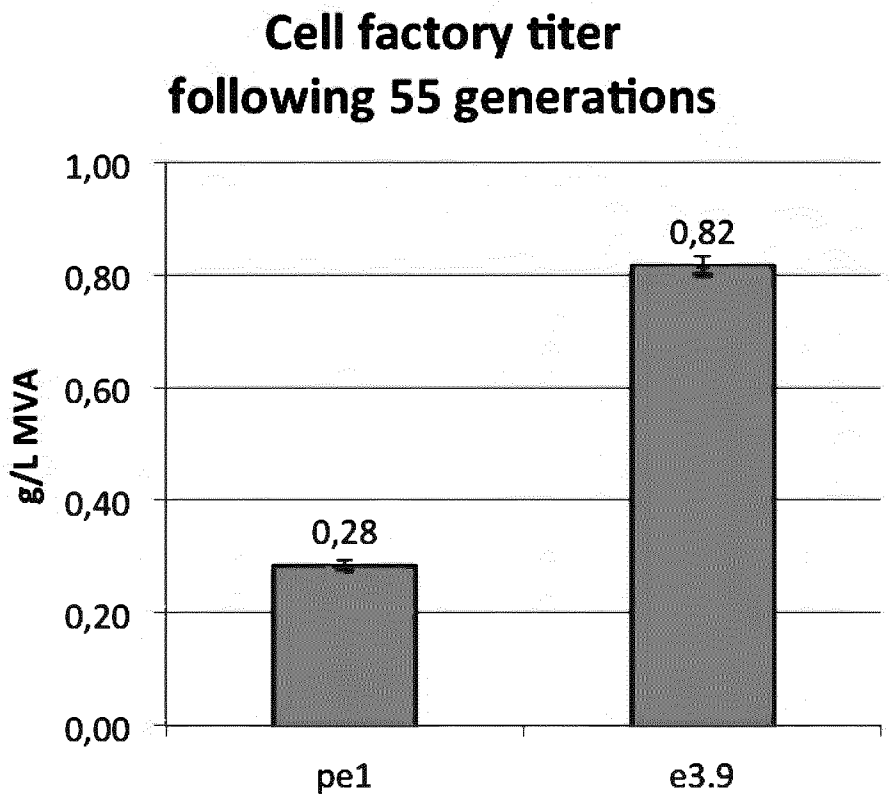


Figure 19


Cell factory titer
following 55 generations
g/L MVA
1,00
0,80
0,60
0,40
0,20
0,00
0,28
0,82
pe1
e3.9

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/073132

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68 C12N15/10 C12N1/20 C12P1/04
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ZHOU LI-BANG ET AL: "Exploring Lysine Riboswitch for Metabolic Flux Control and Improvement of L-Lysine Synthesis in Corynebacterium glutamicum", ACS SYNTHETIC BIOLOGY, vol. 4, no. 6, June 2015 (2015-06), pages 729-734, XP002753291, the whole document -----	1-15
A	WENDISCH VOLKER F: "Microbial production of amino acids and derived chemicals: Synthetic biology approaches to strain development", CURRENT OPINION IN BIOTECHNOLOGY, vol. 30, December 2014 (2014-12), pages 51-58, XP002753292, page 51 - right-hand column, paragraph 2 -----	1-15

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 November 2016	02/12/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Ury, Alain

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2012/153142 A2 (UNIV MANCHESTER [GB]; DIXON NEIL [GB]; MICKLEFIELD JASON [GB]) 15 November 2012 (2012-11-15) the whole document	1-15
A	WO 2015/044456 A1 (UNIV DANMARKS TEKNISKE [DK]) 2 April 2015 (2015-04-02) the whole document	1-15
A	HOWARD SALIS ET AL: "Engineering Bacterial Signals and Sensors", CONTRIBUTIONS TO MICROBIOLOGY, S. KARGER AG, CH, vol. 16, 1 January 2009 (2009-01-01), pages 194-225, XP008120843, ISSN: 1420-9519 the whole document	1-15
A	WO 2012/087483 A1 (UNIV ARIZONA [US]; MELLATA MELHA [US]) 28 June 2012 (2012-06-28) the whole document	1-15
A	WO 2015/118541 A1 (GAVISH GALILEE BIO APPL LTD [IL]) 13 August 2015 (2015-08-13) the whole document	1-15
A	GUZMAN LM ET AL: "Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD Promotor", JOURNAL OF BACTERIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 177, no. 14, 1 July 1995 (1995-07-01) , pages 4121-4130, XP002121022, ISSN: 0021-9193 the whole document	1-15

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/073132

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 2014213779 A1	31-07-2014
		WO 2012153142 A2	15-11-2012
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WO 2012087483 A1	28-06-2012	US 2013337013 A1	19-12-2013
		WO 2012087483 A1	28-06-2012
WO 2015118541 A1	13-08-2015	NONE	